THE UNIVERSITY OF ALBERTA

STUDIES OF THE REPLICATION AND ASSEMBLY OF MENGO VIRUS

by

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A THESIS

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To Linda

for her love and

moral support

over the years.

53S particles are converted to structures having a significantly larger sedimentation coefficient. A similar conversion of 53S to larger particles occurs when the former are purified by equilibrium centrifugation in a CsCl density gradient. The sedimentation coefficient of this new particle has been estimated to be 75S.

Molecular weight determinations of the 14S, 53S and 75S particles by means of Sepharose 4B exclusion chromatography have revealed that the molecular compositions of these particles are $(\varepsilon\alpha\gamma)_5$, $(\varepsilon\alpha\gamma)_{25}$ and $(\varepsilon\alpha\gamma)_{50}$ respectively. Based on this information, a new hypothesis regarding the mechanism of Mengo virus assembly has been proposed in which the viral RNA interacts with either a 75S particle or two 53S particles to form a complex represented by $RNA(\varepsilon\alpha\gamma)_{50}$, before assembly is completed by the addition of two 14S subunits.

The synthesis of viral ribonucleates and polypeptides has been studied in cultured L cells infected with either of two RNA^- ts mutants of Mengo virus (ts 135 and ts 520). It was found that the synthesis of all three species of viral RNA (ss, RF and RI) is inhibited irreversibly and to about the same extent when ts 135-infected cells are shifted from the permissive (33°) to the non-permissive (39°) temperature. Investigations of the *in vivo* and *in vitro* stability of the viral RNA polymerase produced in ts 135-infected cells have shown that the RNA^- phenotype of the mutant reflects a temperature sensitive defect in the enzyme.

Studies of the synthesis of virus-specific polypeptides in ts 135-infected cells have revealed that the post-translational cleavage of structural polypeptide precursors A, B and ε is at least partially blocked at 39°. This defect does not appear to result solely from the

inhibition of viral RNA synthesis at 39°, since cordycepin, which inhibits viral RNA synthesis efficiently, does not block the normal cleavage of polypeptides A and B in wt Mengo-infected cells. The cleavage defect can also be demonstrated in a cell-free system and it appears to result from an alteration (mutation) in the structural polypeptide precursors A and B. The evidence suggests that ts 135 is a double mutant. On the other hand, mutant ts 520 also exhibits an RNA^- phenotype at 39°, but the cleavage of polypeptides A and B in ts 520-infected cells is not blocked at this temperature.

Finally, 53S particles have been shown to accumulate in ts 520-infected cells, but not in ts 135-infected cells, when cultures are shifted from 33° to 39°. This observation provides supporting evidence for the proposal that the 53S particle is an intermediate in the assembly pathway of Mengo virions.

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TABLE OF CONTENTS

(Continued)

TABLE OF CONTENTS (Continued)

(Continued)

(Continued)

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF ILLUSTRATIONS

(Continued)

LIST OF ILLUSTRATIONS (Continued)

LIST OF ABBREVIATIONS

EMC virus	-	encephalomyocarditis virus
ME virus	-	Maus-Elberfeld virus
FMDV	-	foot-and-mouth disease virus
ss RNA	-	single-stranded RNA
ds RNA	-	double-stranded RNA
RF-RNA	-	replicative form RNA
RI-RNA	-	replicative intermediate form RNA
mRNA	-	messenger RNA
VP	-	viral protein
VPg	-	viral gene protein
HS	-	horse serum
BME medium	-	basal minimum essential medium
PBS	-	phosphate buffer saline
RSB	-	reticulocyte standard buffer
TBS	-	tris buffer saline
SDS	-	sodium dodecyl sulfate
PAGE	-	polyacrylamide gel electrophoresis
FPA	-	p-fluorophenylalanine
TPCK	-	tolylsulfonyl-phenylalanyl-chloromethyl ketone
TLCK	-	tolylsulfonyl-lysyl-chloromethyl ketone
PMSF	-	phenylmethylsulfonyl fluoride
MW	-	molecular weight
PFU	-	plaque-forming unit
moi	-	multiplicity of infection
cpm	-	counts per minute

rpm	-	revolutions per minute
uv	-	ultraviolet
g	-	centrifugal force relative to gravity
mA	-	milliampere
mM	-	millimolar
µl	-	microlitre
µCi	-	microcurie
µg	-	microgram
wt	-	wild-type
ts	-	temperature-sensitive

CHAPTER I

GENERAL INTRODUCTION

Picornavirus Classification

In 1963, the International Enterovirus Study Group adopted the term picornavirus (pico = small;rna = ribonucleic acid) to define a group of small, RNA-containing viruses distinguished by (i) their small size (less than 300 Å in diameter), (ii) the absence of an envelope, and (iii) their single-stranded RNA genomes. Although there are plant and bacterial viruses which, on the basis of these criteria, could be classified as picornaviruses, the term has been applied to mammalian viruses only.

All picornaviruses share a fundamental similarity with respect to gross morphology and mode of replication. However, differences have been observed with regard to pH stability and buoyant density in solutions of cesium salts (Andrewes and Pereira, 1972; Newman *et al.*, 1973; Scraba and Colter, 1974), and, based on these parameters, picornaviruses have been further classified into five subgroups as shown in Table 1.

The inclusion of caliciviruses as a subgroup has been questioned since their larger size and characteristic capsid morphology (Zwillenberg and Bürke, 1966; Almeida *et al.*, 1968; Wawrzkiewicz *et al.*, 1968) are atypical of picornaviruses. Furthermore, these viruses have been found to possess only one major capsid protein (Bachrach and Hess, 1973; Burroughs and Brown, 1974), as opposed to other picornaviruses which contain four distinct capsid polypeptides (Rueckert, 1971; Fenner *et al.*, 1974), and to exhibit a different strategy of replication (Ehresmann and Schaffer, 1977). For these reasons, it has been suggested that the caliciviruses be reclassified as a separate family, the Caliciviridae (Burroughs and Brown, 1974; Ehresmann and Schaffer, 1977).

TABLE 1

Picornavirus Classification Scheme

Subgroup	Viruses	Distinguishing Characteristics
Enterovirus	Polio (3 serotypes) Coxsackie A (24 serotypes) Coxsackie B (6 serotypes) Echo (∿ 35 serotypes) Enteroviruses of various animals	Buoyant density (in CsCl) = 1.33-1.35 g/ml Virions stable pH 3-10 Empty capsids produced *in vivo*
Cardiovirus	EMC (Encephalomyocarditis) ME (Maus-Elberfeld) Mengo Columbia-SK	All are antigenically related Buoyant density = 1.34 g/ml Virions stable at low pH, but dissociate at $5 < pH < 7$ in the presence of Cl^- or Br^- ions No stable empty capsids produced *in vivo*
Rhinovirus	Human rhinovirus (∿ 90 serotypes) Equine and bovine rhinoviruses	Buoyant density = 1.38-1.45 g/ml Virions dissociate at pH < 5 Some varieties produce empty capsids *in vivo*
FMDV	Foot-and-mouth disease virus (7 serotypes)	Buoyant density = 1.43 g/ml Virions dissociate at pH < 6.5 Empty capsids produced *in vivo*
Calicivirus	Vesicular exanthema (pigs) Feline calicivirus San Miguel sea lion virus	Buoyant density = 1.37 g/ml Virions dissociate at pH < 4 Slightly larger (35-40 nm diam.) than the above viruses; show cup-like capsomeres when negatively stained

Modified from Scraba and Colter, 1974. The various picornavirus subgroups also have distinctive pathological properties. These are discussed in detail by Andrewes and Pereira (1972). Echo is an abbreviation for "enteric cytopathic human orphan".

Properties of the Virion

Extensive studies on the physical and hydrodynamic properties of picornaviruses, as examplified by Mengo virus (Scraba *et al.*, 1967), have shown that the virion is composed of a molecule of single-stranded RNA (30% by weight) enclosed in a shell of protein (70%). It has a hydrated diameter of about 300 Å (Rueckert, 1971; Fenner *et al.*, 1974; Scraba and Colter, 1974), a sedimentation coefficient ($S^{\circ}_{20,w}$) of 150-160S, a diffusion coefficient ($D^{\circ}_{20,w}$) of 1.44 - 1.47 x 10^{-7} cm^2/sec, and a partial specific volume ($\bar{v}$) of 0.68 - 0.70 ml/g. On the basis of these values, one can calculate that the virion has a particle weight of 8.3 - 8.5 x 10^6 daltons (Scraba *et al.*, 1967).

Structural Components of the Virion

The Viral RNA

The molecular mass of picornaviral RNA has been estimated to be about 2.4 - 2.7 x 10^6 daltons by gel electrophoresis and velocity sedimentation studies (Tannock *et al.*, 1970; Ziola and Scraba, 1974), and by electron microscopy (Granboulon and Girard, 1969; Ziola and Scraba, 1974). Since it contains no unusual nucleotides and approximately equimolar amounts of adenylate, cytidylate, guanylate and uridylate (Newman *et al.*, 1973), it may be calculated that an RNA chain with a mass of 2.6 x 10^6 daltons contains about 7600 nucleotide residues. A number of early studies showed that RNA extracted from the virion is infectious (Colter *et al.*, 1957; Alexander *et al.*, 1958; Huppert and Saunders, 1958; Franklin *et al.*, 1959; Bachrach *et al.*, 1964).

Many viral and animal cellular mRNAs have polyriboadenylic acid, poly A, covalently attached to the 3' terminus (Brawerman, 1974; Shatkin,

1974). In the case of picornaviruses, poly A tracts 50 to 100 nucleotides in length have been found in the RNAs of poliovirus (Yogo and Wimmer, 1972; Spector and Baltimore, 1975a), rhinovirus (Nair and Owens, 1974; MacNaughton and Dimmock, 1975), and foot-and-mouth disease virus (Chatterjee *et al.*, 1976). The cardioviruses EMC (Gillespie *et al.*, 1973; Burness *et al.*, 1977), Mengo (Miller and Plagemann, 1972; Spector and Baltimore, 1975b) and Columbia -SK (Johnston and Bose, 1972) also contain poly A tracts but they seem to be shorter (estimates range from 15-70 residues). It has been suggested that the 3'-terminal poly A is genetically coded and not synthesized by post-transcriptional addition (Yogo *et al.*, 1974; Yogo and Wimmer, 1975; Dorsch-Häsler *et al.*, 1975). Although the biological function of poly A remains obscure, it appears to be essential for the infectivity (Burness *et al.*, 1975; Goldstein *et al.*, 1976) and the efficient *in vitro* translation (Hruby and Roberts, 1977) of the viral RNA.

The 5' end of the picornaviral RNA has also received considerable attention since the discovery that the 5'-termini of most viral as well as cellular mRNAs are blocked and methylated, i.e., "capped" by the structure, m^7G (5') ppp (5') Np (Shatkin, 1976) which appears to be required for the translation of mRNAs in a cell-free system (Muthukrishnan *et al.*, 1975 and 1976). Such a structure is absent in both the picornaviral RNA and its mRNA (Fernandez-Munoz and Darnell, 1976; Hewlett *et al.*, 1976; Nomoto *et al.*, 1976; Shatkin *et al.*, 1976). However, the 5'-terminus of the picornavirus genome RNA has recently been found to be covalently linked to a small protein VPg (Lee *et al.*, 1977; Flanegan *et al.*, 1977; Nomoto *et al.*, 1977a; Sanger *et al.*, 1977; Hruby and Roberts, 1978). Since VPg is also present in replicative intermediate

RNA and on the 5' end of negative RNA strands, it has been suggested that this protein is involved in an early step of RNA synthesis (Flanegan *et al.*, 1977; Nomoto *et al.*, 1977a). The finding that the viral polysomal mRNA, which is not encapsidated and is thus not a precursor for virions, lacks VPg (Nomoto *et al.*, 1977b) has also led to the speculation that this protein may play a role in virus assembly.

The cardioviruses Mengo and EMC, as well as FMDV, also contain tracts of poly C 100 to 200 nucleotides long located near the 5' end of their RNAs (Porter *et al.*, 1974; Perez-Bercoff and Gander, 1977; Rowlands *et al.*, 1978). The function of such a homopolymeric region is unknown and its significance questionable as it is not found in the RNAs of members of the entero- and rhinovirus subgroups (Brown *et al.*, 1974).

The Virion Proteins

Amino acid composition analyses of the total protein from several different picornaviruses gave very similar data (Rueckert, 1971). Few sulfur-containing amino acid residues (2-3 mole %) are present. On the other hand, the proteins have a relatively high content of proline (6-8 mole %) and other non-α-helix-forming residues (valine, isoleucine, serine, threonine and glycine), - an observation compatible with the results of optical rotary dispersion and circular dichroism studies (Scraba *et al.*, 1967; Kay *et al.*, 1970) which indicated that the capsid polypeptides have a low (5-10%) α-helical content.

There is little doubt now that the picornavirus protein capsid is composed of four major polypeptide species with average molecular weights of 33,000 29,000 25,000 and 7,500, deduced mainly from polyacrylamide gel electrophoresis, gel filtration in the presence of

guanidine hydrochloride, and amino acid composition analysis (Rueckert *et al.*, 1969; Stoltzfus and Rueckert, 1972; Burness *et al.*, 1974; Ziola and Scraba, 1974 and 1975). In addition, one or two minor polypeptide components are present in the capsid (Scraba and Colter, 1974). There are approximately 60 copies of each of the major polypeptides and one or two copies of each of the minor polypeptides per virion (Scraba and Colter, 1974).

Morphology and Architecture of the Virion

The structural arrangement of the various polypeptide chains within the virion has been examined by various methods, including electron microscopy, X-ray diffraction, and controlled chemical degradation (Rueckert, 1971).

Electron microscopy of negatively stained preparations of virus particles has not been very useful in elucidating picornavirus architecture since the capsids of these viruses are extremely compact and essentially impermeable to the heavy metal salts commonly used as negative stains. As a result, widely differing estimates [32 - (Mayor, 1964); 42 - (Agrawal, 1966) and 60 - (Horne and Najington, 1959)] of the number of capsomeres per virion have been made by different workers.

From earlier X-ray diffraction studies of poliovirus crystals, Finch and Klug (1959) had concluded that the virion possesses icosahedral (5:3:2) symmetry, and that the capsid is composed of 60 structurally equivalent asymmetric units, each 60 to 65 Å in diameter. It was not possible, however, to define the precise arrangement of these subunits in the virion.

The present knowledge of picornavirus capsid architecture has come largely from studies by Rueckert and co-workers (Rueckert *et al.*, 1969; Dunker and Rueckert, 1971) who, instead of examining the intact virion, studied the products formed by dissociation of the virus under controlled conditions. It has been shown that when the cardioviruses ME (Rueckert *et al.*, 1969; Dunker and Rueckert, 1971), EMC (McGregor *et al.*, 1975), and Mengo (Mak *et al.*, 1970) are incubated at pH 5 to 6.5 in the presence of 0.1 to 0.2 M chloride ions, viral capsids are dissociated into subunits which have sedimentation coefficient of about 14S and a molecular weight of about 425,000. The viral RNA, together with the smallest capsid polypeptide, δ, are released during the dissociation. Polyacrylamide gel electrophoresis revealed that the 14S subunit produced from ME virus is composed of equimolar amounts of the capsid polypeptides α, β and γ with molecular weights of 33,000 30,500 and 25,000 respectively (Rueckert *et al.*, 1969). The 14S subunit can be further dissociated into 5S fragments (MW = 86,000) with the same polypeptide composition by treatment with 2M urea, suggesting that the 5S subunit is a protomer containing one molecule of each polypeptide, i.e. (αβγ), and that the 14S structure is an oligomer composed of five of the smaller 5S subunits, i.e. $(\alpha\beta\gamma)_5$. The capsid of the ME virion is thus made up of 60 protomers (5S) bonded together in clusters of five to form twelve 14S subunits (capsomeres), one of which is centered at each vertex of the icosahedral particle (Dunker and Rueckert, 1971). A very similar model, based on the results of similar types of studies as well as on electron microscopic data, has been proposed for the Mengo virion by Mak *et al.*, (1974).

Although the gross morphology of the picornavirus capsid has been elucidated, the fine detail with respect to the spatial relationships and interactions between individual capsid polypeptides awaits further clarification. For example, the exact location of the smallest capsid polypeptide δ, is still the subject of some controversy. It has been suggested that δ is located on the external surface of the virion and is responsible for its attachment to susceptible cells (Breindl, 1971; Philipson *et al.*, 1973). However, recent studies involving lactoperoxidase-catalyzed iodination of surface polypeptides (Carthew and Martin, 1974; Lund *et al.*, 1977), ^{3}H-acetic acid anhydride treatment of intact virions (Lonberg-Holm and Butterworth, 1976), and immunological tests with monospecific antisera to individual capsid polypeptides (Lund *et al.*, 1977) suggest that this polypeptide may occupy some internal site. Also, knowledge regarding the arrangment of the α,β and δ polypeptides in the structural unit (protomer) is still very limited. From the results of chemical cross-linking studies, Hordern *et al.*(1978) have suggested that these polypeptides occupy discreet domains within the structure unit, that they are held together by α-γ-β or γ-α-β noncovalent interactions, and that the associations of structural units into pentamers are maintained through α-α interactions.

Viral Replication

Attachment, Uncoating and Penetration of the Virion

The first step of the replicative cycle is the attachment of virus particles to specific receptors on the surface of susceptible cells. It has been estimated that each cell possesses 10^{4} to 10^{5} receptors of a particular type (Crowell *et.al.*,1971; Lonberg-Holm and Korant, 1972).

The nature of the virus-receptor interaction appears to be exceedingly complex and has not yet been fully analyzed. However, Angel and Burness (1977) recently suggested that virus attachment depends upon the presence of sialic acid residues on the cell surface and the total number of receptors available per cell.

It has been reported by several workers that the small capsid polypeptide, δ(VP4), is responsible for attachment. Crowell and Philipson (1971) and Lonberg-Holm and Korant (1972) showed that both Coxsackie and rhinoviruses, after elution from cells to which they had been briefly adsorbed, were not capable of reattachment and were thus no longer infectious. Loss of infectivity in these cases and in analogous studies with poliovirus (Breindl, 1971) was correlated to the loss of VP4(δ), which led to the suggestion that this polypeptide may be the "attachment protein". However, the finding that VP4 (δ) occupies an internal location in the virion (Lund *et al.*, 1977) is clearly incompatible with this model. An alternative model has been provided by the studies of Korant *et al.*, (1975) which show that the elution of picornaviruses from cells (with attendant loss of infectivity) is accompanied by a drastic change in the conformation of the capsid polypeptides, and suggest that the loss of VP4 may be incidental to the inactivating process. These workers have shown that preparations of rhinovirus may be resolved into two populations by isoelectric focusing, and that although both populations have the same polypeptide composition (including VP4), those particles whose low isoelectric point suggests an altered conformation of capsid polypeptides do not attach to susceptible cells.

A rearrangement of capsid conformation during or after attachment to the plasma membrane appears to be essential for the subsequent

uncoating process which leads to the release of viral RNA into the cytoplasm. This view is supported by the observation that agents such as glutathione and sodium dodecyl sulfate (SDS) which stabilize the native conformation of poliovirus (Lonberg-Holm *et al.*, 1975) and rhinovirus (Lonberg-Holm and Noble-Harvey, 1973) respectively, as evidenced by increased heat stability, also inhibit plaque formation.

Precisely what follows immediately after the initial capsid modification step is not clear. Based on the observed lipophilic character of modified particles when exposed to artifically prepared liposomes (Lonberg-Holm *et al.*, 1976), Lonberg-Holm and Whiteley (1976) have proposed that the modified virion intercalates into the cell membrane before the final uncoating stage. This model has been extended recently by De Sena and Mandel (1977) who showed that modified virions prepared *in vitro* by incubation with a cell membrane suspension became sensitive to proteases and detergents, and suggested that uncoating involves the occurrence of a number of transitory unstable intermediates. Although the exact mechanism of uncoating is still to be resolved, the final outcome of this process is the release of viral RNA into the cytoplasm.

Alterations of Cellular Metabolism

Shortly after picornavirus infection, the rate of synthesis of cellular proteins, RNA and DNA begins to decline (for a review, see Rekosh, 1977). Although this inhibitory phenonmenon has been recognized for years, elucidation of the mechanism of this viral function has been difficult, partly due to the fact that the rate and extent of such inhibition depend on the multiplicity of infection as well as on the strain of virus and the cell type involved.

Inhibition of host protein synthesis can be attributed to one or more of the following: (i) input virion proteins, (ii) input virion RNA and (iii) a product of a functional viral genome. Over the years reports favoring each possibility have been presented, but evidence that a functional viral genome is required for the inhibition of cellular protein synthesis has been the most compelling. It was first shown by Baltimore and co-workers (1963) that p-fluorophenylalanine (FPA), an inhibitor of protein synthesis, could block the ability of Mengo virus to inhibit cell protein synthesis. Later results have shown that light inactivation of proflavin-sensitized virus (Holland, 1964) or ultraviolet light inactivation of virus (Penman and Summers, 1965; Helentjaris and Ehrenfeld, 1977) prevents it from carrying out the inhibition. Such observations lend strong support to the hypothesis that inhibition of cell protein synthesis requires the activity of a specific viral gene product.

An alternative model has been proposed by Ehrenfeld and Hunt (1971) who suggested that double-stranded (ds) RNA, a by-product of viral replication (see next section), might be responsible for the inhibition of host protein synthesis. This suggestion was based on the observation that double-stranded RNA inhibits the initiation of protein synthesis when added to a rabbit reticulocyte cell-free system. It has been proposed that such inhibition is caused by the inactivation of the initiation factor IF-3 (Kaempfer and Kaufman, 1973). However, it was found later (Celma and Ehrenfeld, 1974) that both viral and cellular mRNA translation are equally sensitive to such inhibition. This result, in conjunction with previous observations that the shutoff of host cell protein synthesis occurs early in infection (Baltimore, 1969) as well

as in the absence of RNA replication (Holland, 1964) when little, if any, ds RNA is present, make it seem unlikely that ds RNA plays a role in the shutoff phenomenon.

Inhibition of cellular protein synthesis is characterized by a disintegration of host cell polysome structures, followed by formation of virus-specific polysomes and virus-directed protein synthesis (Penman *et al.*, 1963). Pre-existing cell mRNA appears not to be degraded after infection (Willems and Penman, 1966; Colby *et al.*, 1974). Leibowitz and Penman (1971) have suggested that shutoff is due to more efficient initiation of translation of viral RNA than of cellular mRNA in infected cells. Using cell-free protein synthesizing systems, Colby *et al.*, (1974) and Lawrence and Thach (1974) demonstrated that viral RNA can suppress the translation of nonviral mRNAs under certain conditions, and concluded that competition between host and viral mRNAs at the initiation step of protein synthesis is probably the basis of such differential translation. Although this argument may explain the inhibition of host protein synthesis late in infection, it does not provide a completely satisfactory explanation for the decrease in host protein synthesis early in infection. Attempts to identify an early viral protein which may function as a positive or negative modulator of protein synthesis have proved unfruitful (Abreu and Lucas-Lenard, 1976). However, Fernandez-Munoz and Darnell (1976), noting that host mRNA has a "capped" 5' end whereas viral mRNA does not, have suggested that this difference may provide a molecular basis for discrimination at the level of initiation between host and virus mRNA .

In addition to cell protein synthesis, host RNA synthesis is also affected early in infection (Baltimore, 1969). Since the rates of RNA

degradation appear to be normal in picornavirus-infected cells (Colby *et al.*, 1974), inhibition seems to occur at the level of RNA synthesis. In an attempt to analyze the molecular mechanism for this shutoff, Schwartz *et al* (1974) and Apriletti and Penhoet (1974) independently demonstrated that nuclei isolated from picornavirus-infected cells showed inhibition of RNA polymerase II activity prior to inhibition of enzymes I and III. However, solubilized enzyme extracts prepared from such nuclei were found to be fully active with exogenous DNA as template. Thus no firm conclusion can be drawn from these studies. The mechanism by which host cell RNA synthesis is shut off remains obscure and the possibility that the phenomenon is secondary to the inhibition of host protein synthesis has not been entirely excluded.

Inhibition of DNA synthesis during picornavirus infection has also been ascribed to the shutoff of cell protein synthesis (Hand *et al.*, 1971) since this effect can be mimicked by antibiotic-mediated inhibition of protein synthesis. Recently Hand and Oblin (1977) showed that Mengovirus infection blocks the entry of dTTP into DNA by inhibiting the initiation of synthesis of new DNA chains and have suggested that this may represent a separate, more specific mechanism by which DNA synthesis is inhibited.

Replication of Viral RNA

Kinetic studies have shown that the synthesis of viral RNA is initiated within half an hour of infection and that viral RNA accumulates at an exponential rate for the next 3-4 hours, after which synthesis becomes linear (Baltimore, 1969) and eventually stops at about 7-8 hours post-infection. The fate of the newly synthesized viral RNA is mediated by some as yet unknown regulatory mechanism which

determines whether the new molecule is to be encapsidated as virion RNA, to serve as a template for transcription, or to function as a mRNA for translation.

Knowledge regarding the precise mechanism of RNA replication has come largely from studies on RNA bacteriophages (for reviews see Weissmann *et al.*, 1968 and 1973; Spiegelman *et al.*, 1968) as well as on picornaviruses (for review see Levintow, 1974). The key to our understanding has been the elucidation of the structure of another RNA species found in infected cells, - the replicative intermediate (RI). In the picornavirus system, the RI is heterogeneous in size and has a sedimentation coefficient ranging between 20 and 70S in sucrose density gradients. Physicochemical characterization of the RI indicated that it consists of a double-stranded "core" with several single-stranded "tails" of variable lengths attached (Baltimore *et al.*, 1966; Baltimore, 1968). That this structure is a true intermediate in viral RNA synthesis was first suggested by the observation that it is preferentially labeled by brief pulses of radioactive precursors. The transcribing role of RI was further confirmed by *in vitro* pulse-chase experiments using a membrane-bound enzyme preparation (Girard, 1969; McDonnell and Levintow, 1970), in which the radioactivity in pulse-labeled RI was shown to flow eventually into single-stranded progeny RNA molecules. The mode of replication has been shown to be largely semiconservative. The picture that has emerged is one in which a parental viral RNA molecule (plus strand) serves as template from which 4-6 strands of complementary RNA (minus strands) are transcribed, - each displacing a pre-existing minus strand from the duplex as its synthesis proceeds. The complementary RNA molecules in turn serve as templates for the synthesis of viral RNA

molecules (plus strands) in an analogous fashion. This model suggests the existence of two different classes of RI - one involved with the synthesis of minus strands and the other with the synthesis of plus strands. However, synthesis of RNA is asymmetric in that more plus than minus strands are made, an observation consistent with the finding that most of the complete single-stranded molecules in RI's are complementary (minus strand) RNA (Bishop *et al.*, 1969). Whether this is a result solely of the removal of plus strand RNA for translation or encapsidation is not known.

In addition to single-stranded RNA and the replicative intermediate, a third species of RNA termed the "replicative form" (RF) has been found in picornavirus-infected cells (Baltimore and Girard, 1966). Physico-chemical studies have revealed that it is a double-helical molecule consisting of one viral (+) strand and one complementary (-) strand. Since RF has been found to accumulate in the infected cell at a relatively constant rate, and to bear no demonstrable precursor-product relationship with viral RNA (Baltimore, 1968; Girard, 1969), it is believed to be a by-product of the replication process and to have no significant biological role.

The observation that viral RNA synthesis takes place in the cytoplasm of infected cells rather than in the nucleus (Franklin and Baltimore, 1962), coupled with the fact that metabolic inhibitors which prevent DNA-directed DNA and RNA synthesis have no effect on the multiplication of picornaviruses (Simon, 1961; Reich *et al.*, 1962), led to the suggestion that the synthesis of viral RNA is mediated by an RNA-dependent RNA polymerase. The presence of such a virus-specific enzyme (replicase) was subsequently demonstrated in both Mengo- and poliovirus-

infected cells (Baltimore and Franklin, 1963; Baltimore *et al.*, 1963). As isolated from cytoplasmic extracts, the enzyme activity is associated with a membrane-bound structure composed mainly of protein and nucleic acids and termed the "replication complex" (Girard *et al.*, 1967). In the presence of all four ribonucleoside triphosphates, such a complex can carry out *in vitro* reactions in which the products are qualitatively analogous to those synthesized in the infected cell (Horton *et al.*, 1966).

Early attempts to identify the replicase polypeptide(s) were hampered by the lack of a suitable procedure to purify soluble enzyme preparations active enough to respond to an exogenously supplied template (Arlinghaus and Polatnick, 1969; Ehrenfeld *et al.*, 1970). However, a poly C-dependent RNA polymerase was isolated from EMC virus-infected cells (Rosenberg *et al.*, 1972). SDS-polyacrylamide gel analysis of the purified enzyme complex revealed the presence of five polypeptides (MW = 72,000 65,000 57,000 45,000 and 35,000), one of which has a molecular weight (57,000) identical to that of the viral non-structural polypeptide E. The other four polypeptides, believed to be of host origin, have molecular weights almost identical to those of the four subunits of Qβ replicase (Kondo *et al.*, 1970; Kamen, 1970). Similar analyses of RNA replicase preparations from Mengo- and poliovirus-infected cells (Loesch and Arlinghaus, 1975; Lundquist *et al.*, 1974), together with further work on the EMC virus system (Traub *et al.*, 1976) have provided additional circumstantial evidence in support of the hypothesis that the virus-specified, non-structural polypeptide E is a component of the viral-specific RNA polymerase.

Attempts to demonstrate replicase activity in cell-free systems using exogenous templates have met with only limited success. However, it is of interest to note that recently Flanegan and Baltimore (1977) have detected a poly A-oligo U-dependent poly U polymerase in poliovirus-infected HeLa cells, and have suggested that the viral RNA polymerase may be a primer-dependent enzyme.

Synthesis of Viral Protein

The synthesis of all virus-specific proteins takes place on large (350S) polysomes (Penman *et al.*, 1964) which are tightly bound to cytoplasmic membranes (Caliguiri and Tamm, 1969). Only one type of messenger RNA is present in these polysomes and it is indistinguishable from the virion RNA in terms of size, base composition and ability to hybridize to minus strands (Levintow, 1974). That the virion RNA can function as mRNA has also been demonstrated repeatedly in cell-free systems where the polypeptides synthesized are the same as those found in infected cells (Rekosh, 1977).

From gel electrophoretic analysis of extracts of picornavirus-infected cells, approximately 14 virus-specific polypeptides can be identified (for review see Hershko and Fry, 1975). A study of the kinetics of labeling of these polypeptides revealed that the smaller capsid and non-capsid polypeptides are formed by the cleavage of larger precursors and that the combined molecular weights of the stable final products correspond to the coding capacity of the entire viral genome (Summers and Maizel, 1968; Holland and Kiehn, 1968; Jacobson and Baltimore, 1968a).

Jacobson and Baltimore (1968a) first suggested that the viral RNA is translated to yield a large precursor polypeptide ("polyprotein"),

from which all viral proteins are produced by subsequent cleavages. With the exception of Coxsackie virus-infected cells (Kiehn and Holland, 1970), such a large polyprotein (MW > 200,000) cannot be detected under normal conditions, probably due to the fact that cleavage of the nascent polyprotein occurs while it is still being synthesized. However, the presence of this giant molecule can be demonstrated easily in infected cells in which proteolytic cleavage is blocked by the incorporation of amino acid analogues into the primary sequence of the protein (Jacobson *et al.*, 1970; Paucha *et al.*, 1974), by specific protease inhibitors such as TPCK or TLCK (Korant, 1972; Summers *et al.*, 1972)or by zinc ions (Butterworth and Korant, 1974; Korant and Butterworth, 1976). Accumulation of giant polypeptides has also been observed in cells infected with certain temperature-sensitive mutants and incubated at the restrictive temperature (Cooper *et al.*, 1970; Garfinkle and Tershak, 1971). Furthermore, Roumiantzeff *et al.* (1971) reported that membrane-bound polysomes from poliovirus-infected cells synthesized a protein *in vitro* whose size suggested that it was produced by uninterrupted translation of the entire viral genome.

Indirect evidence to support the above hypothesis has also been provided by studies of picornavirus RNA-directed protein synthesis in cell-free systems, which indicate that the viral RNA molecule contains only a single site for the initiation of translation (Öberg and Shatkin, 1972; Boime and Leder, 1972; Smith, 1973; Villa-Komaroff *et al.*, 1975). These observations would lead one to predict that *in vivo*, all picornavirus-specified proteins would be synthesized in equimolar amounts, and this appears to be so in the case of EMC virus (Butterworth and Rueckert, 1972a; Butterworth, 1973). However,

contradictory findings have been reported. For example, Celma and Ehrenfeld (1975) have reported findings which they interpret as evidence that the poliovirus genome contains two initiation sites for translation, and Lucas-Lenard (1974) and Paucha *et al.*(1974) have shown that at later times in the Mengo virus replication cycle, there is an increase in the amount of capsid relative to non-capsid protein synthesized. The latter results suggest that Mengo RNA may contain a weak internal termination signal, as seems to be the case in some RNA bacteriophages (Weissmann *et al.*, 1973).

The presently known cleavage steps in the formation of picornavirus polypeptides are shown in Fig. 1. The gene order of the stable viral proteins, namely - δ,β,γ,α,G,H,F,I and E, has been determined mainly by the use of pactamycin, a drug which at appropriate concentrations specifically inhibits the initiation of protein synthesis but not the elongation of peptide chains (Summers and Maizel, 1971; Butterworth and Rueckert, 1972a). Other techniques that have been applied to establish precursor-product relationships include kinetic pulse-chase experiments (Butterworth and Rueckert, 1972b) and cyanogen bromide and tryptic mapping of isolated polypeptides (Butterworth *et al.*, 1971; Dobos and Plourde, 1973). All such studies have shown that the pattern of post-translational cleavage is very similar for poliovirus (Butterworth, 1973), EMC virus (Butterworth and Rueckert, 1972b; Butterworth, 1973) rhinovirus 1A (Butterworth, 1973; McLean and Rueckert, 1973) and Mengovirus (Paucha *et al.*,1974; Lucas-Lenard, 1974).

As depicted in Fig. 1, the primary cleavage of the nascent polyprotein gives rise to three large primary polypeptides, A, F and C, as well as two smaller, noncapsid polypeptides (G and H). Product C,

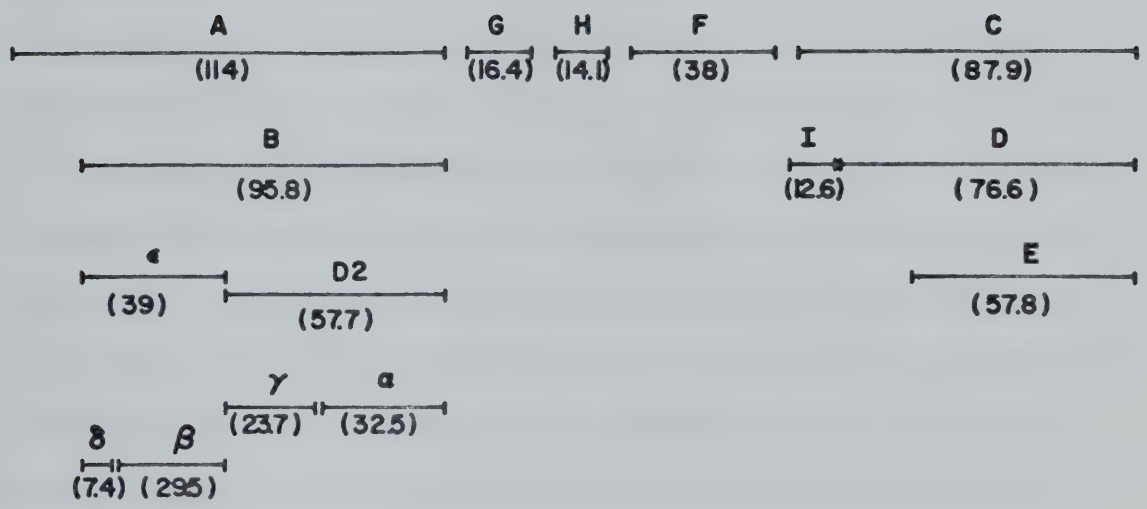


Figure 1. Cleavage scheme of Mengo virus-specific polypeptides. The numbers in brackets indicate the molecular weights, in thousands, of the polypeptides.

corresponding to the 3' region of the viral RNA, undergoes further proteolytic cleavages to produce the stable, noncapsid polypeptides E and I. Product A, which corresponds to the 5' region of the viral RNA,is the precursor of the capsid polypeptides. The conversion of A → ε, α and γ occurs by what have been termed secondary cleavages. The morphogenic cleavage, ε → δ + β, occurs during the final stages of virion assembly, apparently after viral RNA is encapsidated. Polypeptide F is stable and does not undergo further cleavages.

Although such posttranslational cleavages are clearly enzymatic processes, the precise nature of the enzyme(s) involved has not been established. Available information has come largely from studies of the effects of protease inhibitors on the cleavage process. Korant (1972), working with type 2 poliovirus, showed that the chymotrypsin inhibitor TPCK blocks the cleavage of the polyprotein in infected monkey kidney cells, but that the trypsin inhibitor TLCK is more effective in infected HeLa cells. Similar findings have also been reported by Summers *et al.*, (1972). The premise that the primary cleavages are catalyzed by a cellular protease (or proteases) has been further supported by the observation (Korant, 1972) that the poliovirus polyprotein, isolated from monkey kidney cells infected in the presence of TPCK, can be converted, by incubation with extracts of uninfected cells, into products similar to those produced by primary cleavages *in vivo*. In contrast, secondary cleavages were shown to occur only in the presence of extracts of infected cells, which suggests that a viral protease is involved at this stage (Korant, 1972; Korant, 1973).

Based on information regarding the N- and C-terminal amino acids of various picornavirus capsid proteins, Ziola and Scraba (1976) have

suggested that the secondary cleavages, $B \rightarrow \varepsilon + D_2$ and $D_2 \rightarrow \gamma + \alpha$, are probably carried out by a single virus-specified protease with a specificity for peptide bonds involving the carbonyl function of glutamine residues.

A different approach, adopted by Lawrence and Thach (1975), has been to examine the synthesis and cleavage of polypeptides in a cell-free system. Their findings suggest that an early stage in the processing of the capsid precursor A requires a proteolytic activity which is not detected in uninfected control cells and which appears to co-purify with the viral capsid protein γ. Since γ is an integral part of A, such a cleavage process would be classified as autocatalytic. These investigators have also proposed that polypeptide γ may be responsible for the maturation cleavage, $\varepsilon \rightarrow \delta + \beta$, that takes place during the final assembly of the virion. In this case, cleavage could be initiated by the addition of viral RNA.

Virion Assembly

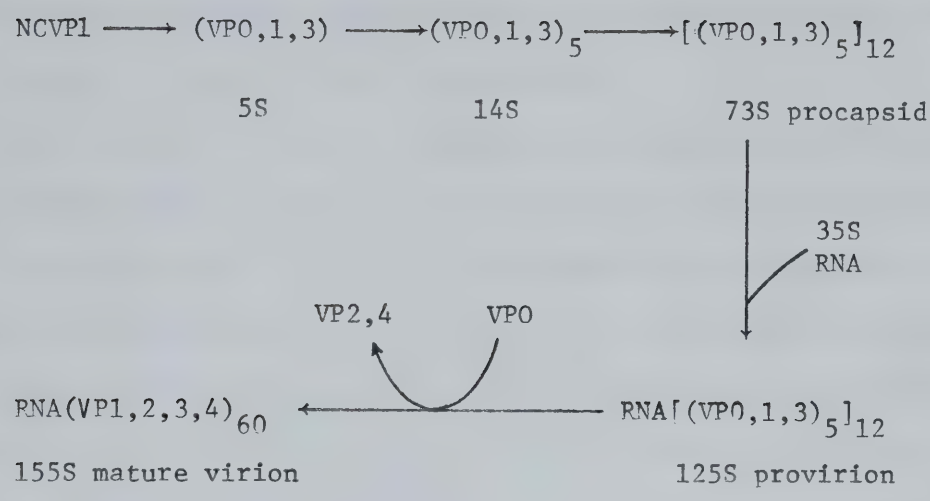
Studies on the morphogenesis of picornaviruses have focused primarily on the identification and characterization of capsid-related structures in extracts of infected cells (for a review see Casjens and King, 1975). Such structures, termed "subviral particles", represent immature virions in various stages of assembly. In the case of poliovirus, subviral particles having sedimentation coefficients of 5S, 14S, 73S and 125S have been detected in infected cells, in addition to 155S mature virions.

The 5S particle is a complex of VP0, VP1 and VP3 (ε, α and γ in the cardiovirus system) and probably corresponds to the "immature protomer" which arises immediately upon cleavage of the precursor of the capsid

proteins (Phillips *et al.*, 1968). Although a kinetic precursor-product relationship has never been demonstrated, the size and composition of the 14S particle is consistent with the proposition that it is an aggregate of five of the 5S units. The 14S subunits are capable of self-assembly *in vitro* to form a 73S structure which is indistinguishable from the naturally occurring empty capsid (Phillips, 1969 and 1971). This reaction is enhanced by the addition of rough membranes from infected cells (Perlin and Phillips, 1973) and it has been suggested that the membranes promote the assembly process by adsorbing and concentrating the reactants.

The 73S particles, which are produced during the normal course of poliovirus infection (Maizel *et al.*, 1967), also contain VP0, VP1 and VP3, and on the basis of electron microscopic studies, are generally believed to be empty capsids (procapsids). Jacobson and Baltimore (1968b) found that 73S particles accumulate in infected cells in the presence of 3mM guanidine hydrochloride, and since the labeled protein was found to be chased into mature virions upon removal of the guanidine, they concluded that the 73S particle is a precursor in the assembly process. Fernandez-Tomas and Baltimore (1973) subsequently detected a 125S particle which contains RNA, but in which VP0 is still uncleaved. The 125S particle, or "provirion", thus appears to be the immediate precursor of the mature virion whose capsid contains VP1, VP2, VP3, VP4 and only a small amount of VP0, the immediate precursor of VP2 and VP4.

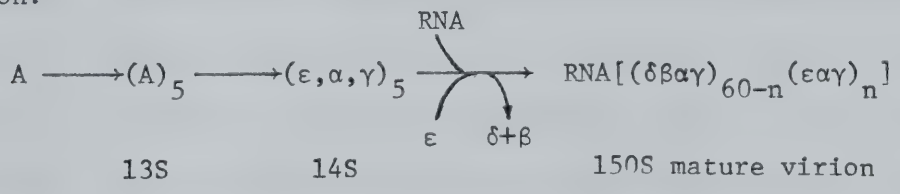
On the basis of these data, the following pathway has been suggested for poliovirus morphogenesis (Casjens and King, 1975).



Although this scheme seems to be complete, and compatible with most of the data obtained from studies of the poliovirus system, it has been criticized, - most of the criticism being directed at the ascribed role of the 73S procapsid. Firstly, there is no convincing evidence that empty capsids are produced in cardiovirus systems. Secondly, attempts to demonstrate that the empty capsids found in other picornavirus systems [eg. in cells infected with FMDV (Rowlands *et al.*, 1975) and bovine enterovirus 1 (Su and Taylor, 1976)] are intermediates in the assembly pathway have not been successful. Thirdly, Ghendon *et al.* (1972) reported that 14S rather than 73S particles accumulate in poliovirus-infected MiO cells (from rhesus monkey tonsils) in the presence of guanidine, and that when the guanidine is removed, the accumulated 14S material is chased into virions without the appearance of 73S procapsids. Fourthly, Wright and Cooper (1976) showed that although VP0,VP1 and VP3 are in close association with the replication complex, the probable site of viral RNA encapsidation, they are not present in the form of empty capsids. Considered in toto, these observations do cast

some doubt on the assumption that the 73S particle is a true intermediate in the assembly pathway, and leave open the possibility that it is merely a product of abortive assembly.

Turning to the cardiovirus subgroup, McGregor *et al.*(1975), working with EMC virus reported the detection of two capsid precursors (13 and 14S) in infected cells. The 13S particle, believed to be of polypeptide composition $(A)_5$, is rapidly labeled when infected cells are incubated with ^3H- amino acids, and its radioactivity is chased after a short period into the 14S particle of polypeptide composition $(\varepsilon,\alpha,\gamma)_5$. Since these workers could not detect $(\varepsilon,\alpha,\gamma)$ in monomeric form in their cell extracts, they have suggested that polymerization of A chains may be a prerequisite for secondary cleavages, and on that basis have proposed the following scheme for the assembly of the EMC virion:



Later work by the same group (McGregor and Rueckert, 1977) indicated that the early steps in the assembly of rhinovirus 1A follows a similar pathway.

Whether or not the two pathways outlined above reflect real fundamental differences in assembly mechanisms among various picornavirus subgroups is debatable. For example, it is possible that the initial steps in the assembly of poliovirus also involve a 13S aggregate, and that the 5S structures are merely breakdown products of the 14S particle. Also, Prather and Taylor (1975) have reported the detection of 80 and 125S particles during the replication of Mengo virus in a

restrictive bovine kidney cell line (MDBK). However, no attempt was made to characterize these structures. In view of such ambiguities, it is clear that additional studies, ideally on individual members of each subgroup, are required before the full details of the assembly pathway(s) of the picornaviruses are elucidated.

* * * * * * *

The work described in this thesis may be divided into two parts: 1) a study of a subviral particle having a sedimentation coefficient of about 50S, which had been detected earlier in this laboratory (Paucha, 1976) in extracts of Mengo virus-infected mouse L cells, - the primary objective being to establish whether this particle, which had not been described previously, is an intermediate in the morphogenesis of Mengo virions. 2) A detailed examination of two of the temperature-sensitive mutants of Mengo virus produced in this laboratory (Downer *et al.*, 1976), undertaken as part of a larger investigation aimed at gaining an understanding of the biological functions of the non-structural, virus-specified polypeptides synthesized in infected cells.

The study of the "50S particle" has provided rather convincing evidence that it is an intermediate in the assembly of Mengo virion, and, on the basis of its biochemical and biophysical properties, a new hypothesis regarding the mechanism of Mengo virus assembly has been formulated. The investigation of the temperature-sensitive mutants revealed that while both are temperature-sensitive with respect to RNA replication, one contains a second lesion that results in a partial block in the cleavage of the large capsid precursors. These studies also provided supporting evidence for the proposed role of the "50S particle" in virus assembly.

CHAPTER II

ROUTINE MATERIALS AND METHODS

Media

Tissue Culture Media

Eagle's minimum essential medium (MEM) for suspension cultures and Eagle's basal medium (BME) for monolayer cultures were obtained in powder form from the Flow Laboratories, Rockville, Md. The powder was dissolved in distilled, deionized water and sodium bicarbonate was added to a final concentration of 0.12% before sterilization by Millipore filtration (0.22 µm pore size - Millipore Corp., Bedford, Ma.). Both media were supplemented with the following materials immediately before use:

1) Horse serum (Flow Laboratories) to a final concentration of 5% for growth of cells or 1% for production of virus.
2) Penicillin G (Glaxo-Allenbury's Ltd., Toronto, Ont.) and Streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) at final concentrations of 100 I.U. and 50 µg/ml respectively.

Amino Acid Deficient Medium

The medium is similar to Eagle's basal medium (BME) in composition except that it contains no amino acids other than glutamine. After addition of sodium bicarbonate (final conc. = 0.06%), the medium was sterilized and supplemented with 1% horse serum and antibiotics as described above.

Virus Diluent

This is the buffered (pH 7.6) balanced salt solution (PBS) described by Dulbecco and Vogt (1954) containing 0.2% bovine plasma

albumin, fraction V (Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y.), 0.02% phenol red (J.T. Baker Chemical Co., Phillipsburg, N.J.), 100 I.U./ml penicillin and 100 µg/ml streptomycin.

Overlay Diluent

This solution contains three times the normal concentration of Hanks' salts, six times normal concentrations of both Basal Eagle's amino acids (Baltimore Biological Laboratory, Division of Becton Dickinson and Company, Cockeysville, Md.) and MEM vitamin solution (Gibco), five times the usual concentrations of penicillin and streptomycin, 0.78% sodium bicarbonate and 30% inactivated (56°C for 45 min) calf serum.

Agar Overlay

This is prepared by mixing one volume of overlay diluent with two volumes of 1.6% distilled water solution of Noble agar (Difco Laboratories, Detroit, Mich.) at 45°C.

Cultured L Cells

Earle's L-929 strain of mouse fibroblasts (Sanford *et al.*, 1948) were used as host cells in these studies. They were obtained from the American Type Culture Collection, Rockville, Md.

The cells were maintained in monolayer culture in 1-litre Blake bottles (Kimble Products, Owens-Illinois Co., Toledo, Ohio) at 37°C. When the cultures had reached confluence, the cells were harvested by a brief incubation with trypsin (0.25%, Difco) in a buffer containing 10 mM phosphate, pH 7.4, 142.8 mM sodium chloride and 2.8 mM potassium chloride. Cells from several bottles were resuspended in fresh BME - 5% HS and used to maintain the Blake bottle stock, while the remaining

cells were resuspended in spinner medium at a concentration of 2×10^5 cells/ml and transferred to 1- or 2-litre spinner flasks (Bellco Biological Glassware, Viniland, N.J.) for further propagation at 37°C.

Virus

The plaque variant of Mengo virus designated as M-Mengo (Ellem and Colter, 1961) was used throughout these studies.

Virus Growth in Roller Bottles

Confluent L cell monolayers were grown in large cylindrical bottles (490 mm x 110 mm diameter - Bellco Biological Glassware) coated with fetal calf serum (Flow Laboratories) prior to seeding to facilitate the attachment of cells. L cells harvested from suspension culture were then resuspended in fresh BME-5% HS at a density of about 10^6 cells/ml and dispensed in 150 ml aliquots to the coated roller bottles. The bottles were rotated on a Bellco roller apparatus at 0.2 rpm for 3 hr after which the speed was increased to 1 rpm. When confluent monolayers were obtained, the cells in each roller bottle were infected at an approximate multiplicity of 10 PFU/cell with Mengo virus suspended in 20 ml of growth medium (1% horse serum). The bottles were then rotated at 0.2 rpm for 3 hr. and 1 rpm for an additional 20 to 24 hr. At the end of this time, most of the cells had lysed and could be dislodged from the glass by shaking.

Virus Purification

The procedure employed for virus purification has been described by Ziola and Scraba (1974).

Lysates of infected L cells were pooled and centrifuged at 1,000 g for 15 min. The pellet was resuspended in a small volume of distilled

water, frozen and thawed three times to release trapped virus and re-centrifuged. The combined supernatants were chilled in ice, after which the virus was precipitated by the addition of cold (-20°) methanol to a final concentration of 20% and allowing the solution to stand overnight at -20°C. The precipitate was collected by centrifugation at 5,000 g for 30 min, suspended in 0.2 M sodium phosphate buffer, pH 7.8 and homogenized by hand in a Potter-Elvehjem tissue homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.) to disrupt large aggregates. A solution of α-chymotrypsin (3x recrystallized - Worthington Biochemicals, Freehold, N.J.) was added to give a final enzyme concentration of 0.8 mg/ml and the mixture incubated for 20 min at 37° with stirring. An equal volume of 0.2M sodium pyrophosphate, pH 8.0 was added and incubation continued for an additional 20 min. The mixture was then chilled and clarified by centrifugation at 20,000 g for 10 min. Virus was pelleted from the supernatant by centrifugation at 120,000 g for 60 min, and resuspended by homogenization in a small volume of 0.1 M sodium phosphate buffer, pH 7.4.

The partially purified virus was sedimented through a discontinuous gradient made of equal (13 ml) volumes of 15% and 30% sucrose by centrifugation for 20 hr at 20,000 rpm in a spinco SW27 rotor. The pellet was again resuspended by homogenization in 0.1 M sodium phosphate, pH 7.4, and mixed with an aqueous solution of Cs_2SO_4 to a final density of 1.31 g/cm^3. After centrifugation for 20 hr at 40,000 rpm in a Spinco SW50.1 rotor, the virus, which appeared as a white band near the middle of the gradient, was collected through the side of the tube with a syringe. Salt was removed by passage through a 3 cm^2 x 20 cm column of Sephadex G25 and the virus was stored at 4°C in 0.1 M sodium phosphate

buffer, pH 7.4

Preparation of Radioactively-Labeled Virus

L cell monolayers in roller bottles were infected with Mengo virus in the amino acid deficient medium described. Two hours after infection, ^{3}H- or ^{14}C-labeled amino acid mixtures (New England Nuclear, Montreal, Que.) were added to give final concentrations of 2 µCi/ml or 0.2 µCi/ml respectively. Subsequent procedures were identical to those already described.

Plaque Assay of Infectious Virus

This procedure has been described by Campbell and Colter (1965). Monolayers of L cells on 60 x 15 mm plastic petri dishes (Falcon Plastics, Oxnard, Ca.) were prepared by harvesting cells from Spinner culture, resuspending at a density of 5×10^{5} cells/ml in fresh growth medium (BME - 5% HS), and adding 5 ml of this suspension to each plate. Confluence was reached after incubation for 24 hr at 37° in a humidified atmosphere of 5% CO_2 in air.

The medium was aspirated and each plate inoculated with 0.1 ml of appropriate dilutions of virus in virus diluent. After incubation for 1 hr at 37°C to allow virus to attach, each plate was overlaid with 4.5 ml of agar overlay.

Plaques were visible after incubation at 37° for an additional 48 hr. To facilitate counting, plates were stained at this time by addition of 3 ml of agar overlay containing 0.01% neutral red (Fisher Scientific, Fair Lawn, N.J.).

CHAPTER III

MORPHOGENESIS OF MENGO VIRUS: IDENTIFICATION AND CHARACTERIZATION OF SUBVIRAL PARTICLES

Introduction

It is generally accepted that all picornaviruses share a fundamental similarity with respect to gross morphology, physical and hydrodynamic properties, composition, and the mechanisms by which their proteins and RNA are synthesized. However, there is no convincing evidence that members of the different subgroups follow a common pathway of virion assembly. Uncertainty in this respect can be attributed to the following factors. Firstly, with the exception of poliovirus, information regarding the presence of subviral particles in picornavirus - infected cells is fragmentary. Secondly, progress has been hampered by the inherent difficulty associated with kinetic pulse-chase experiments designed to establish precursor-product relationships between subviral particles and mature virions.

In the case of poliovirus, the most extensively studied of the picornaviruses, four subviral particles, having sedimentation coefficients of 5S, 14S, 73S and 125S, have been described (Casjens and King, 1975). Although there is little doubt that the 14S structure, with the composition $(VP0,1,3)_5$ - or $(\varepsilon\alpha\gamma)_5$ using the cardiovirus subgroup nomenclature - is a precursor in the assembly pathway of all picornaviruses, the role of the 73S particle (empty capsid) as an intermediate has been questioned, partly because there is no convincing evidence that comparable structures are produced in cells infected with other members of the picornavirus family.

In view of this uncertainty, the discovery of a "50S particle", in addition to the 14S structure, in Mengo virus-infected cells in our laboratory (Paucha, 1976) was of considerable interest. In this chapter, the characterization of the "50S particle" is described in terms of its biochemical and biophysical properties. The results of these studies suggest that this particle may be a true intermediate in the Mengo virus assembly process, and provide the basis for a new hypothesis concerning the morphogenesis of the Mengo virion.

Materials and Methods

Infection of Monolayers and Labeling of Viral Polypeptides

Cells grown in either petri dishes (100 mm diam.) or roller bottles (425 mm x 110 mm diam.) were infected at an estimated multiplicity of 100 PFU/cell with Mengo virus suspended in virus diluent. After incubation for 1 hr at 37°, the monolayers were washed with warm (37°) BME-5% HS and then incubated in the same medium. Cells were pulse-labeled by incubation for either 20 min (petri dish cultures) or 30 min (roller bottle cultures) in amino acid-deficient Eagle's medium containing 1% HS and either [^{3}H] amino acids (30 μCi/ml) or [^{14}C] amino acids (5 μCi/ml). Labeling was done at either 4.5 or 5 hr postinfection in the case of petri dish cultures and at 5.5 hr postinfection with cells grown and infected in roller bottles. In both cases the cultures were incubated in amino acid-deficient medium for the hour immediately preceding the labeling period. When labeling was followed by a chase period, the monolayers (after removal of the radioactive medium) were washed once with PBS and incubated for the desired period of time in BME-5% HS.

Fractionation of Cells and Isolation of Subviral Particles

The cells were harvested from roller bottles by trypsinization, collected into ice-cold BME + 5% HS, pelleted by low-speed centrifugation, and washed sequentially with ice-cold PBS and RSB (reticulocyte standard buffer, 10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$) before being resuspended in homogenizing buffer (1 mM Tris-HCl, pH 7.4, 1 mM KCl, and 1.5 mM $MgCl_2$). The monolayers grown in petri dishes were washed with PBS and RSB, after which the cells were scraped off the dishes into homogenizing buffer.

Cells were homogenized in an all-glass Dounce homogenizer with a tightly fitting pestle, and the tonicity of the homogenate was adjusted to give final concentrations of 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, and 6 mM β-mercaptoethanol (TBS) by adding the appropriate volume of 10 x concentrated TBS. After the addition of Nonidet P40 and sodium deoxycholate to give final concentrations of 1 and 0.5%, respectively, the nuclei were removed by low-speed centrifugation, and the supernatant was separated into cytoplasmic supernatant (S_{20}) and cytoplasmic pellet (P_{20}) as described by Roumiantzeff *et al.*(1971) by centrifugation for 30 min at 20,000 g (13,000 rpm, JA-20 rotor, Beckman J21 centrifuge). Material having a sedimentation coefficient of 14 S or larger was collected from the S_{20} fraction by centrifugation for 5 hr at 45,000 rpm (type 50 rotor; Beckman L5-65 centrifuge). The pellet so obtained is referred to herein as the P_{45} fraction.

Sucrose Density Gradient Analyses

Centrifugal analyses of S_{20} and P_{45} fractions isolated from cells grown in petri dishes were carried out by layering 0.5 to 0.8 ml aliquots on 16 ml linear 15-45% sucrose gradients (in TBS) and

centrifuging them for 13.5 hr at 20,000 rpm (SW 27.1 rotor; Beckman L5-65 centrifuge). The fractions (0.5 ml) were collected using a Beckman gradient fractionator linked to an LKB peristaltic pump and fraction collector. The single analysis of an S_{20} fraction from a roller bottle culture as described here was carried out on a 36 ml 15-45% sucrose gradient. Centrifugation was for 12 hr at 20,000 rpm (Beckman SW 27 rotor), and the gradient was fractionated (1 ml fraction) using an ISCO Model D gradient fractionator equipped with a Model UA-2 uv monitor (Instrumentation Specialties Co. Inc., Lincoln, Nebraska).

Following gradient centrifugation of the S_{20} preparations, 200 µl aliquots of the gradient fractions were applied to filter paper disks (Whatman No. 3, 2.3 cm diameter) which were air-dried and washed sequentially with 10% TCA, 5% TCA, ethanol, and acetone before being placed in scintillation vials. After centrifugation of the P_{45} preparations, 200 µl aliquots of the gradient fractions were added directly to scintillation vials. In both cases, radioactivity was measured in the presence of 10 ml of Aquasol scintillation fluid (New England Nuclear Corp.) in a Beckman liquid scintillation spectrometer (Model LS-230).

Polypeptide Analysis

The polypeptide composition of Mengo virions and of the subviral structures recovered from the sucrose density gradients was determined by SDS-polyacrylamide gel electrophoresis carried out according to the procedure of Weber and Osborn (1969), using 7.5% gels (21 cm long and polymerized in 6 mm i.d. glass tubes) that had been acid-washed and coated with dichlorodimethysilane. The gels were preelectrophoresed for 1 hr at 8 mA/gel before the samples were applied.

Gradient fractions containing labeled virions or subviral particles were made 1.5% in SDS, 2% in β-mercaptoethanol, 10^{-3} M in phenylmethylsulfonyl fluoride (PMSF), and 0.002% in bromophenol blue and were heated at 100° for 5 min before being applied to the gels. Electrophoresis was carried out at 4 mA/gel for the first hour, after which the current was increased to and held at 8 mA/gel for an additional 16 hr. The gels were fractionated using the automatic Aliquogel fractionator (Gilson Medical Electronics, Inc.), and the fractions were incubated overnight at 65° in 0.5 ml of 30% H_2O_2, after which the radioactivity in each was measured as described earlier.

Isolation of *E. coli* Ribosomes

E. coli grown in complete broth medium containing [^{3}H] uracil (5 μCi/ml) were harvested at mid-log phase. The cells were then washed with a cold buffer containing 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, 50 mM KCl, and 6 mM β-mercaptoethanol (TKM) and disrupted by grinding with glass beads in a mortor and pestle. After sedimentation of unbroken cells and debris, the ribosomes were pelleted by centrifugation for 2 hr at 45,000 rpm (type 50 rotor; Beckman L5-65 centrifuge). Adventitiously adsorbed proteins were removed by resuspending the ribosomes in a solution containing equal volumes of TKM buffer and 1M NH_4Cl and holding the suspension at room temperature for 5 min. After removing large ribosomal aggregates by low speed centrifugation, the washed ribosomes were pelleted as described above, resuspended in TKM buffer, and stored at -20°C.

Sepharose 4B Column Chromatography

Sepharose 4B, as a suspension in distilled water, was obtained from Pharmacia Fine Chemicals. After repeated washings in TBS, the

Sepharose 4B was suspended in TBS, poured into a column (80 cm x 1 cm diam.) and packed slowly at 4°C by passing the same buffer through the column at a flow rate of 10-12 ml/hr. Following a further packing period of approximately 7 to 10 days, the void volume of the column, determined as the elution volume of calf thymus DNA, was found to vary by less than 0.5 ml over a period of several weeks.

Chromatography was performed at 4°C. In most cases, the sample whose elution volume (Ve) was to be determined was mixed with 4 OD_{260} units of calf thymus DNA (for determination of void volume, V_o) and approximately 10,000 cpm of ^{14}C-thymidine (for determination of total volume of the gel bed, V_t) to make up a total loading volume of 0.8 ml. The column was eluted with TBS at a flow rate (monitored by means of an LKB peristaltic pump) of 12 ml/hr. Fractions of approximately 1 ml volume were collected. Depending on the sample, aliquots of appropriate fractions were taken for optical density measurements at either 260 or 280 nm, and for radioactivity measurement in Aquasol scintillation fluid as described earlier.

Molecular Weight Determinations of Subviral Particles

Sepharose 4B chromatography data are expressed in terms of Kav, a parameter which is defined as follows:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \qquad [1]$$

where V_e = elution volume of a solute; V_o = void volume of the column; and V_t = total volume of the gel bed.

The Kav of a molecule is in turn correlated with its Stokes radius according to the following equation proposed by Laurent and Killander (1964):

$$(-\log Kav)^{1/2} = \alpha(\beta + a) \qquad [2]$$

where a = Stokes radius, and α and β are constants related to the intrinsic properties of the gel matrix. The validity of these theoretically derived relationships has been confirmed experimentally by Siegel and Monty (1966) who demonstrated a linear relationship between elution volume expressed in terms of Kav and Stokes radius for a variety of proteins.

With a Stokes radius measured by means of a column that has been previously calibrated with markers of known Stokes radii, and a sedimentation coefficient determined by sucrose density gradient centrifugation, reasonable estimates of the molecular weight of a macromolecule can be obtained by employing the following equation:

$$M = \frac{6\ \pi\eta\ Nas}{(1 - \bar{\nu}\rho)} \qquad [3]$$

where M = molecular weight, a = Stokes radius, s = sedimentation coefficient, $\bar{\nu}$ = partial specific volume, η= viscosity of medium, ρ = density of medium, and N = Avogadro's member.

The markers used in the chromatography studies were fibrinogen, [^{3}H] uridine-labeled R17 bacteriophages, [^{14}C] amino acid-labeled Mengo virions and 13.4S subunits prepared therefrom (Mak *et al.*, 1971). The elution volumes (V_e), and hence the Kav, of these markers were thus easily measured by monitoring either the OD_{280} (in the case of fibrinogen) or radioactivity (in the case of R17, Mengo virus and Mengo 13.4S subunit) of appropriate fractions. The Stokes radius of fibrinogen has been reported to be 107 Å (Scheraga and Laskowski, 1957). The Stokes radii of the Mengo virion, the 13.4S Mengo viral subunit and of R17 were calculated by means of Equation 3, using the known molecular weights, sedimentation coefficients and partial

specific volumes of these markers (Table 2).

Chemicals and Radioisotopes

Calf thymus DNA, cordycepin and bovine fibrinogen were purchased from Sigma Chemical Company, St. Louis, Missouri. [^{3}H]-uridine-labeled R17 bacteriophage was a gift from Dr. S. Igarashi of this department. All radioisotopes were purchased from New England Nuclear: [^{3}H] amino acids (1.0 mCi/ml), [^{14}C] amino acids (0.1 mCi/ml), [^{3}H] uridine (24.2 Ci/mmole), [^{3}H] uracil (40-50 Ci/mmole), and [^{14}C] thymidine (54 mCi/mmole).

Results

Identification of Subviral Particles

The data obtained from sucrose density gradient centrifugal analysis of the S_{20} fraction prepared from a roller bottle culture of L cells, which were pulse-labeled for 30 min at 5.5 hr after infection with Mengo virus (m.o.i. ≈ 100) and harvested after a chase period of 60 min, are illustrated in Fig. 2. The fractions were monitored for OD_{260} and for radioactivity. In addition to the intact virions (150 S), a well-defined peak of labeled material was found at a position between those occupied by the 40 and 60 S ribosomal subunits, which are resolved clearly from each other and from 80S ribosomes in a 15-45% sucrose density gradient. From the position of this peak in the gradient, the material contained therein was designated initially "50S particles".

When particulate material present in the S_{20} fraction was sedimented by high-speed centrifugation and the pellet (P_{45} fraction) was analyzed by sucrose density gradient centrifugation, the results shown

TABLE 2

Calculated Stokes Radii of Marker Particles

	Molecular weight ($\times 10^6$)	Sedimentation coefficient ($\times 10^{-13}$ sec)	Partial specific volume (ml/g)	Stokes radius[a] (Å)
Mengo virion	8.32[b]	151[b]	0.70[b]	145
Mengo virus 13.4S subunit	0.428[c]	13.4[d]	0.73[d]	76
R17 phage	3.88[e]	78[e]	0.68[e]	139

[a] Calculated by means of Equation 3.

[b] Scraba *et al.* (1967)

[c] Calculated from the reported composition $(\alpha\beta\gamma)_5$ (Mak *et al.*,1971) and molecular weights of individual polypeptides (Ziola and Scraba, 1974).

[d] Mak *et al.* (1971)

[e] Boedtker and Gesteland (1975)

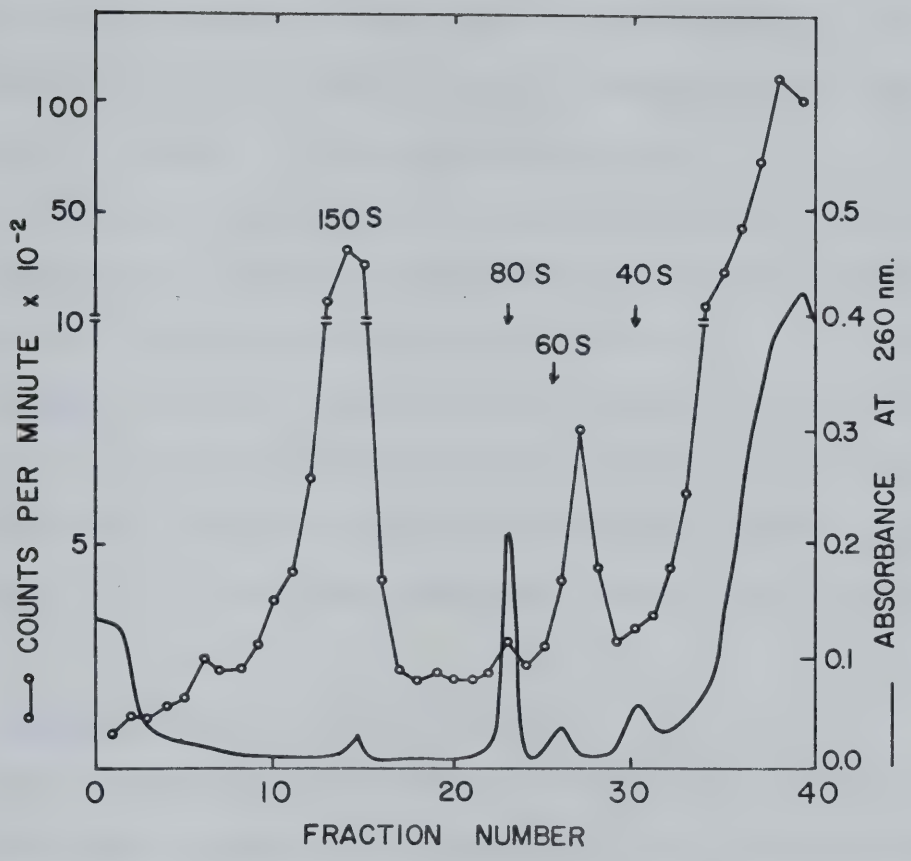


Figure 2. Sedimentation analysis of a cytoplasmic supernatant (S_{20}) prepared from a roller bottle culture of Mengo virus-infected L cells. Cells were labeled for 30 min with [^{3}H] amino acids (30 µCi/ml) at 5.5 hr postinfection, and the S_{20} fraction (in TBS) was isolated after a 60 min chase. Centrifugation through a 36 ml 15-45% linear sucrose gradient was at 20,000 rpm for 12 hr at 4° (Beckman SW 27 rotor). Sedimentation was from right to left. The gradient was fractionated and analysed for acid-insoluble radioactivity (○———○) and absorbance at 260 nm (——).

in Fig. 3 were obtained. In addition to the virus (150S) and "50S"peaks, a well-defined peak of more slowly sedimenting material was observed. On the basis of compositional analysis (see following section) and position in the gradient, it seems clear that the material in this peak is identical to the 14S particles shown by McGregor *et al.* (1975) to be present in extracts of EMC virus-infected cells.

A more precise estimate of the sedimentation coefficient of the "50S particle" was obtained by comparing its sedimentation behavior in 15-45% linear sucrose density gradients with those of *E. coli* ribosomal subunits (50S and 30S). Illustrative data are shown in Fig. 4, in which the positions of L cell ribosomal subunits in identical gradients are also indicated. From the results of these studies, the sedimentation coefficient of this previously undetected subviral particle was calculated to be 53S.

Composition of the Subviral Particles

Peak fractions from each of the 14, 53, and 150S peaks obtained by sucrose density gradient centrifugation of a P_{45} preparation were pooled separately and analyzed by SDS-PAGE. The results illustrated in Fig. 5 show that the 14 and 53S particles contain equimolar amounts of polypeptides ε, α and γ. The mature virions as shown earlier by Ziola and Scraba (1974) contain approximately equimolar amounts of polypeptides α,β,γ and δ plus a small quantity of polypeptide ε,the immediate precursor of β and δ (Butterworth *et al.*, 1971; Paucha *et al.*, 1974).

To determine whether the 53S particle contains any viral RNA, [^{3}H] uridine (10 μCi/ml) was added to a culture of infected cells 1 hr before they were pulse-labeled for 20 min (at 5 hr postinfection)

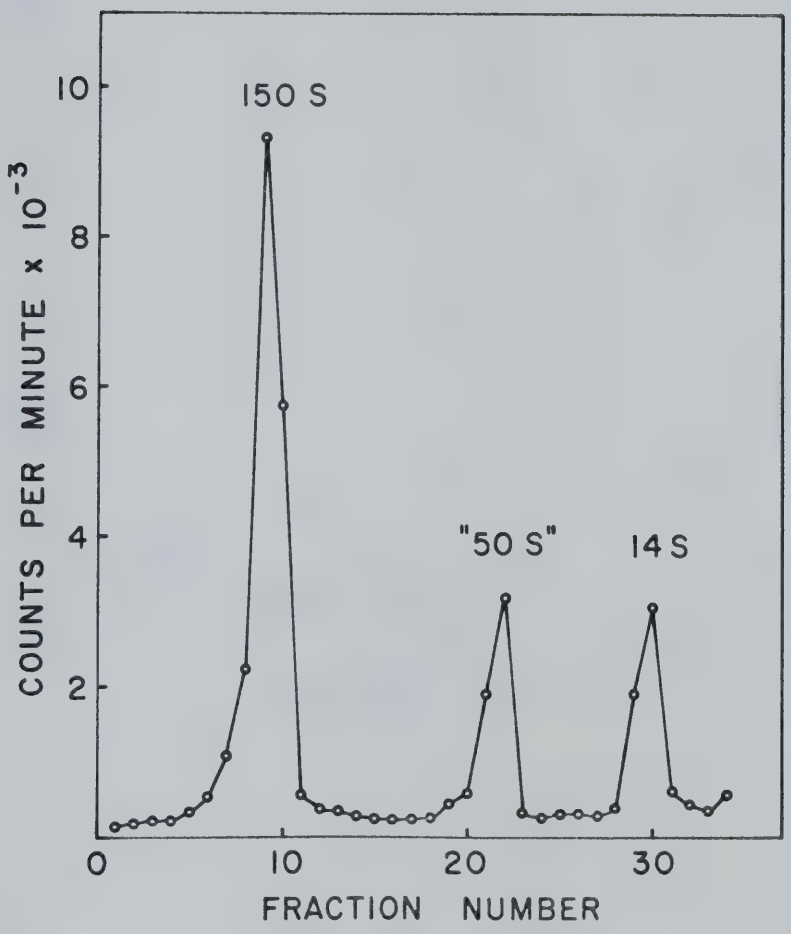


Figure 3. Sedimentation analysis of virus-specific particles isolated from Mengo virus-infected cells. An S_{20} fraction, isolated from a roller bottle culture as indicated in the legend to Fig. 2, was centrifuged for 5 hr at 45,000 rpm at 4° (Beckman type 50 rotor). The pellet (P_{45} fraction) was resuspended in TBS and layered on a 16 ml 15-45% linear sucrose gradient, which was then centrifuged at 20,000 rpm for 13.5 hr at 4° (Beckman SW 27.1 rotor). Sedimentation was from right to left.

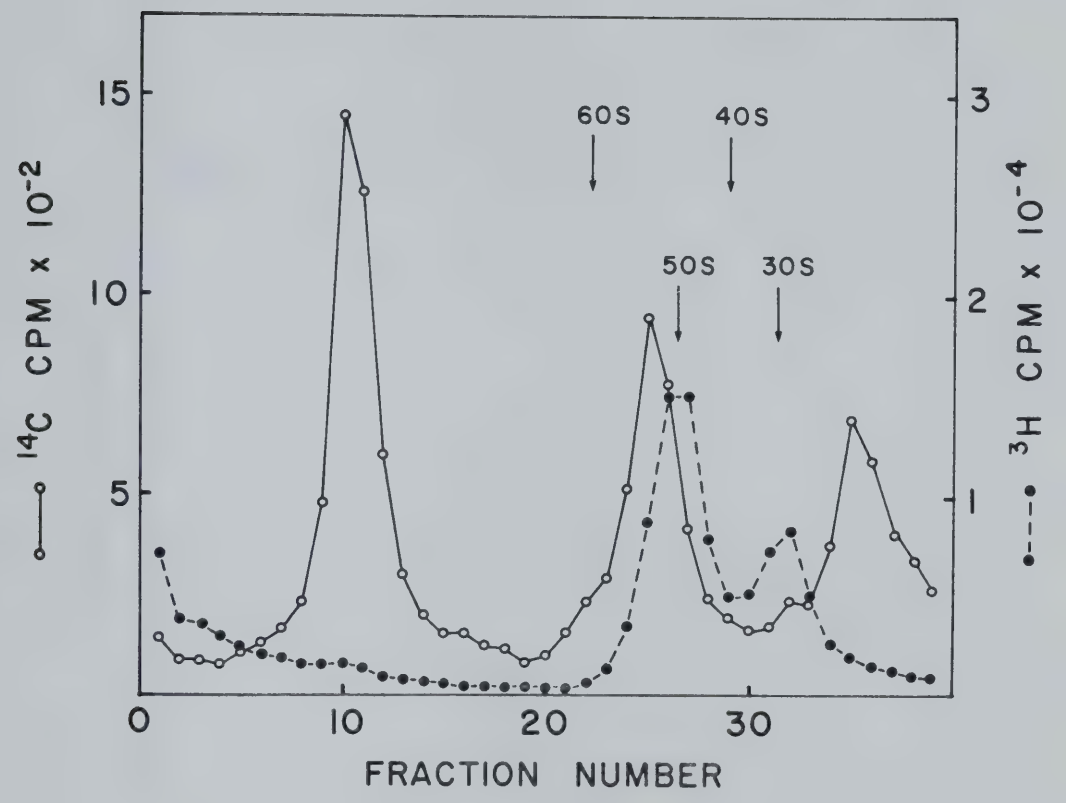


Figure 4. Sedimentation analysis of a P45 fraction isolated from Mengo virus-infected cells pulse-labeled with ^{14}C-amino acids for 20 min at 5 hr postinfection and harvested after a chase period of 30 min. The P45 isolated was resuspended in a low Mg^{++} TBS (0.5 mM $MgCl_2$) to which purified *E. coli* ribosomes (^{3}H-uracil-labeled) were also added. Centrifugation through a 16 ml 15-45% linear sucrose gradient in the same buffer was for 13.5 hr at 20,000 rpm (Beckman 27.1 rotor). Gradient fractions were analyzed for acid insoluble ^{14}C-counts per minute (○) and ^{3}H-counts per minute (●). The positions of L cell ribosomal subunits (40S and 60S) were determined from a parallel gradient in which the P45 fraction from uninfected cells labeled overnight with ^{3}H-uridine was analyzed.

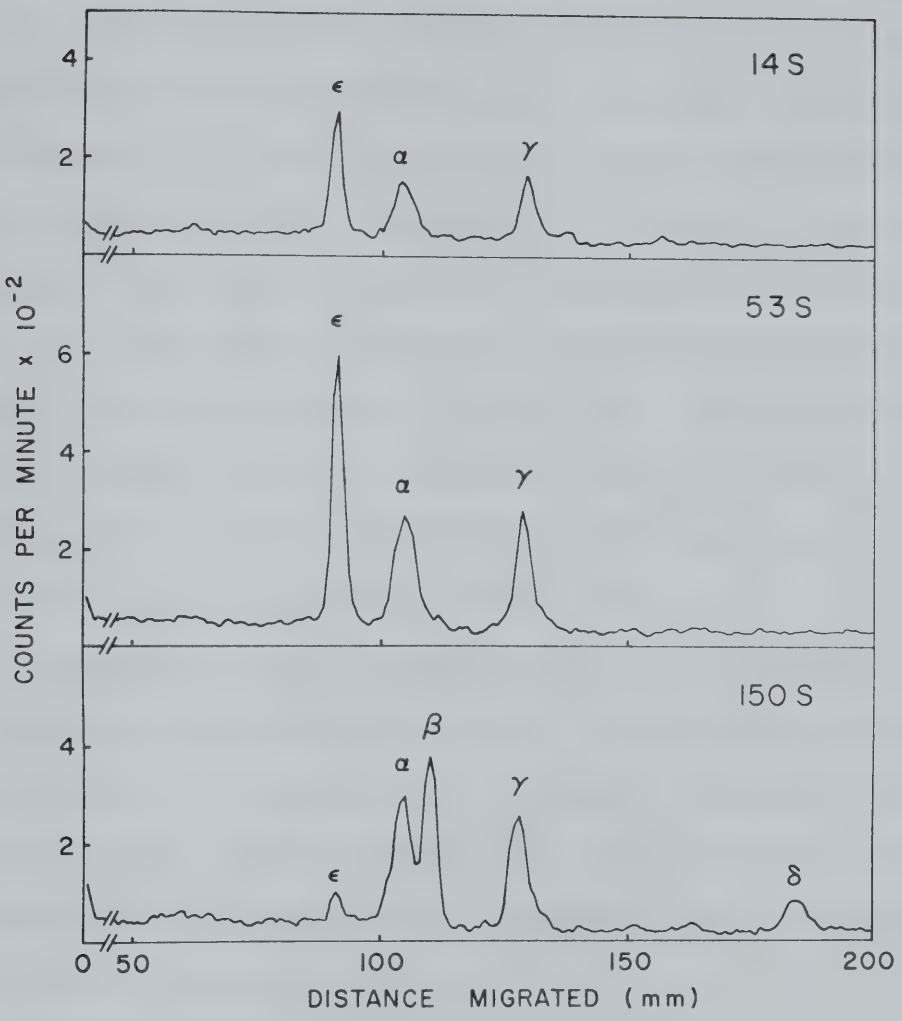


Figure 5. SDS-polyacrylamide gel electrophoretic analysis of 14, 53 and 150S particles. Samples of the peak fractions from the sucrose density gradient illustrated in Fig. 3 were solubilized by heating them in SDS and then analyzed as outlined under Materials and Methods. Migration was from left to right.

with ^{14}C-labeled amino acids (5 μCi/ml). After incubation for an additional 30 min in the absence of labeled amino acids but in the presence of [^{3}H] uridine, the cells were harvested and a P_{45} fraction was isolated therefrom and analyzed by sucrose density gradient centrifugation. The results illustrated in Fig. 6 show clearly that the 53S particle contains no viral RNA. Confirmatory evidence was obtained by centrifuging 53S particles (recovered by high-speed centrifugation from pooled sucrose density gradient fractions) to equilibrium in a CsCl density gradient (Fig. 7). The 53S particle was found to have a buoyant density of 1.296 g/cm^3, which is substantially lower than that of the intact virion (1.330 g/cm^3) but very close to that expected of a particle containing protein only.

Precursor Role of 53S Particles: Pulse-chase Experiments

Since the polypeptide composition of the 53S particle suggested that it might be an intermediate in the assembly of Mengo virions, conventional pulse-chase experiments were carried out in an attempt to demonstrate a precursor-product relationship between the two subviral particles and intact virions.

Replicate monolayer cultures of infected cells were pulse labeled with [^{3}H] amino acids for 20 min at 4.5 hr postinfection, and cytoplasmic supernatants prepared immediately after the labeling period and after subsequent chase periods of 30, 60 and 110 min were analyzed by sucrose density gradient centrifugation. S_{20} rather than P_{45} fractions were used in this study, because the recovery of 14S particles in the P_{45} fraction was found to be less than quantitative. Illustrative data are presented in Fig. 8.

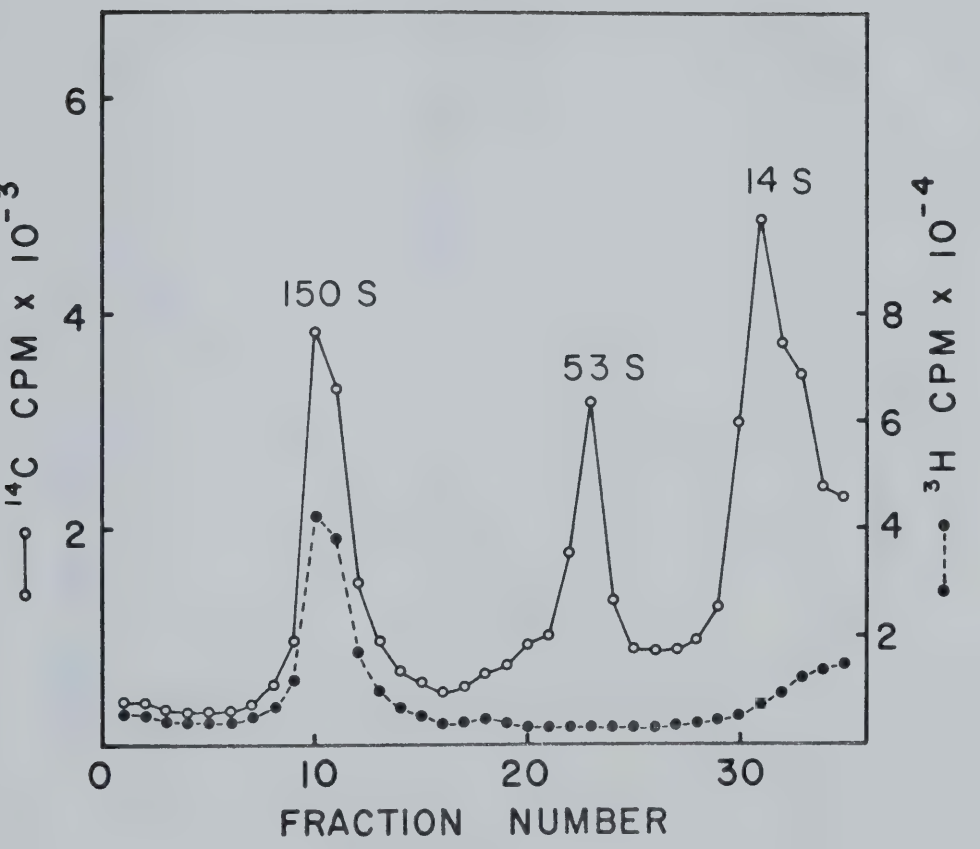


Figure 6. Sedimentation analysis of a P_{45} fraction isolated from Mengo virus-infected cells labeled with [^{3}H] uridine and ^{14}C-amino acids. Cells were labeled with [^{3}H] uridine from 4 to 5 hr post-infection, then pulse labeled for 20 min with ^{14}C-labeled amino acids, and harvested after an additional incubation period of 30 min in medium containing [^{3}H] uridine. Centrifugation through a 16 ml 15-45% linear sucrose density gradient was for 13.5 hr at 20,000 rpm (Beckman SW 27.1 rotor). Gradient fractions were analyzed for acid-insoluble ^{14}C-counts per minute (○) and ^{3}H-counts per minute (●).

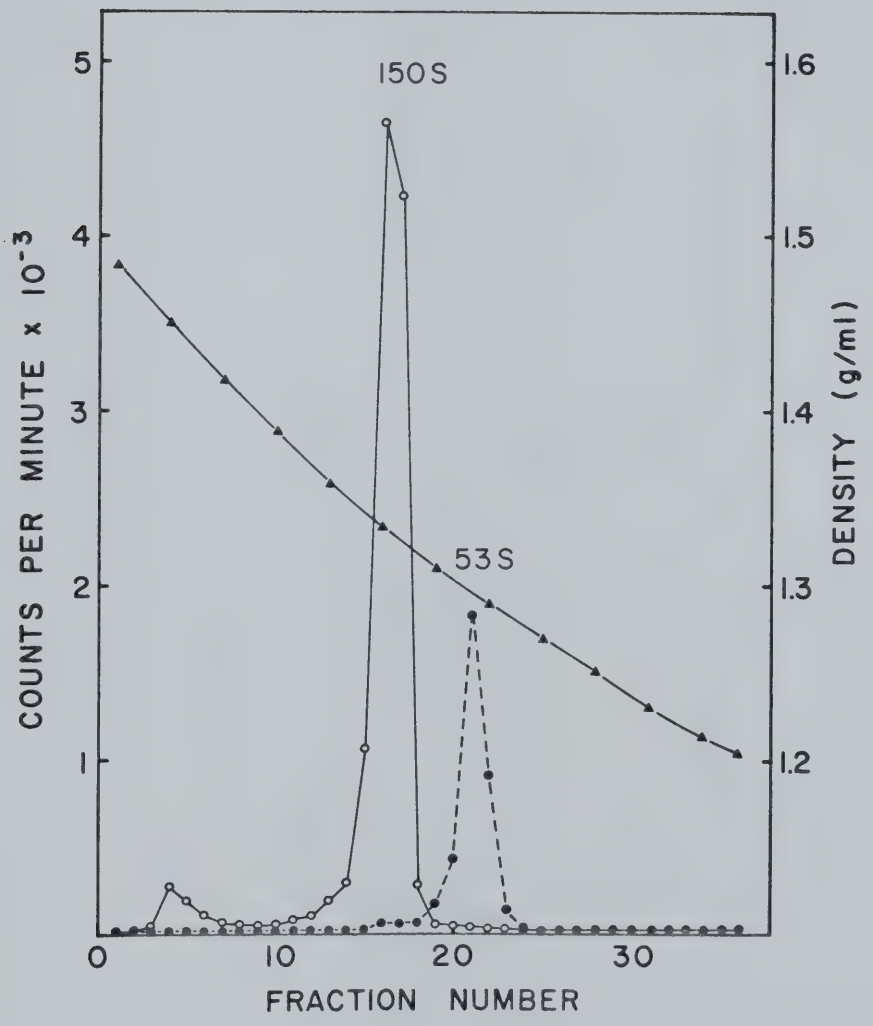


Figure 7. Equilibrium centrifugation of 150S and 53S particles in CsCl density gradients. Samples of the peak fractions from a gradient comparable to that illustrated in Fig. 3 were centrifuged in separate gradients and are superimposed here for comparison. (○) 150S virion fraction; (●) 53S particle fraction; (▲) buoyant density (g/ml).

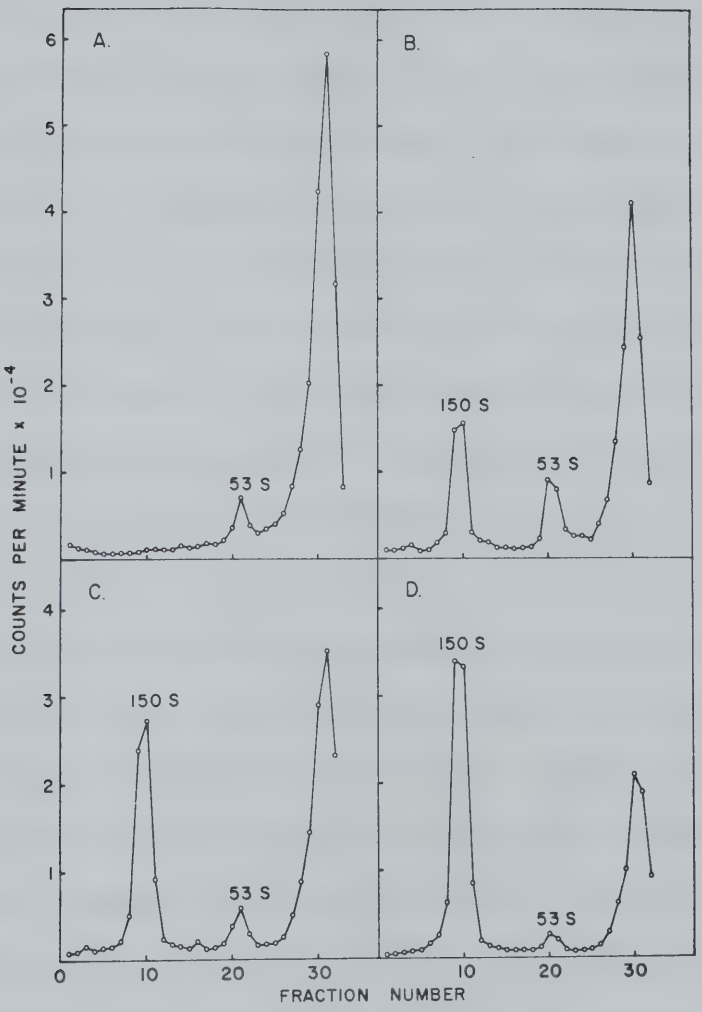


Figure 8. Sedimentation analysis of S_{20} fractions prepared from Mengo virus-infected cells labeled for 20 min with [^{3}H] amino acids at 4.5 hr postinfection. Centrifugation through 16 ml 15-45% linear sucrose density gradients was for 13.5 hr at 20,000 rpm (Beckman SW 27.1 rotor). S_{20} fractions were prepared immediately after the labeling period (panel A) and after chase periods of 30 min (panel B), 60 min (panel C,) and 110 min (panel D).

No labeled virions were produced during a 20 min labeling period (Fig. 8A), but radioactivity was found in the 53S peak and in the top fractions, which contained a mixture of viral polypeptides in addition to the 14S particles. Analysis of the S_{20} fraction prepared after a 30 min chase period (Fig. 8B) revealed the appearance of a virus peak (150S), an increase in the amount of label in the 53S peak, and a loss of radioactivity from the top fractions. With longer chase periods (Figs. 8C and 8D), a progressive and essentially quantitative transfer of radioactivity from both the top fractions and the 53S peak into the virions was observed. Very little of the 53S material remained after a 110 min chase (panel D), and the residual radioactivity in the top fractions reflected the presence of labeled viral, nonstructural proteins (no 14S particles could be detected in the P_{45} fractions prepared after a chase period of 110 min).

These data show clearly that during a chase following a 20 min labeling period, there is a progressive flow of radioactivity from 14S and 53S particles into mature virions. The data suggest but do not prove that the 53S particle is an intermediate between the 14S particles and virions on the assembly pathway. More convincing evidence that this is the case was obtained from similar studies in which the effects of cordycepin were examined.

Effect of Cordycepin on Viral RNA Synthesis and on the Formation of 53S Particles.

Reports that the adenosine analog cordycepin (3'-deoxyadenosine) inhibits the replication of a number of viruses including human rhinovirus (Nair and Owens, 1974), Newcastle disease and Sendai viruses (Mahy *et al.*, 1973), vaccinia virus (Nevins and Joklik, 1975), and

poliovirus (Nair and Panicali, 1976) prompted an examination of the effects of this compound in our system. It was found that cordycepin inhibits the synthesis of viral ribonucleates in Mengo-infected cells and that at a concentration of 200 µg/ml, inhibition is rapid and efficient (85-90%). This observation is illustrated by the data shown in Fig. 9, as is the fact that the inhibition can be relieved simply by removing the medium containing the cordycepin and washing the monolayers twice with normal medium. After removal of the inhibitor, viral RNA synthesis resumes immediately and at a rate comparable to that in control cultures.

The effect of cordycepin on the formation of 53S particles was then examined. Replicate cultures of infected cells were pulse labeled with [^{3}H] amino acids at 4.5 hr postinfection, and cytoplasmic supernatants (S_{20}) were prepared (i) immediately after the labeling period, (ii) after chase periods of 45 and 135 min in BME-5% HS, (iii) after chase periods of 45 and 135 min in BME-5% HS containing 200 µg of cordycepin/ml, and (iv) after a chase period of 45 min in BME-5% HS containing 200 µg of cordycepin/ml followed (after washing) by an additional chase period of 90 min in BME-5% HS. The results of sucrose density gradient analyses of the S_{20} fractions are illustrated in Fig. 10.

The presence of cordycepin during a 45 min chase period was found to reduce sharply the number of mature virions produced and to result in a significant accumulation of 53S particles (Fig. 10B). When cordycepin was removed after a chase period of 45 min, and when the cultures were incubated for an additional 90 min in the absence of the inhibitor, a quantitative transfer of radioactivity from the 53S to the virion peak was observed (Fig. 10C). The profile (not shown)

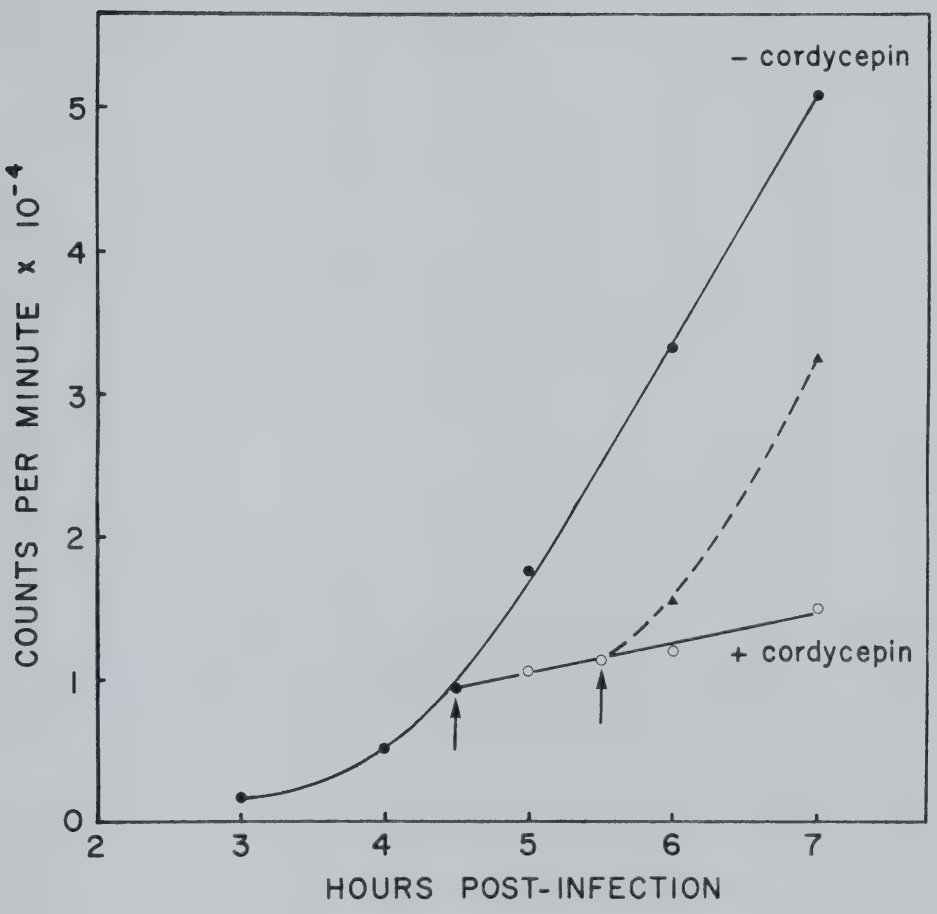


Figure 9. Effect of cordycepin on the synthesis of viral ribonucleates. [^{3}H] Uridine (10 μCi/ml) was added to replicate cultures of Mengo virus-infected cells at 2 hr postinfection. At 4.5 hr postinfection, cordycepin (200 μg/ml) was added to some of the cultures, and at 5.5 hr postinfection, half of the cultures to which cordycepin had been added were washed twice with culture medium and reincubated in medium containing [^{3}H] uridine. At various times from 3 to 7 hr postinfection, the cultures were harvested, the cells were lysed, and the lysates were analyzed for acid-insoluble radioactivity. (●), no cordycepin added; (○), cordycepin added at 4.5 hr postinfection; (▲), cordycepin added at 4.5 hr postinfection and removed by washing at 5.5 hr postinfection.

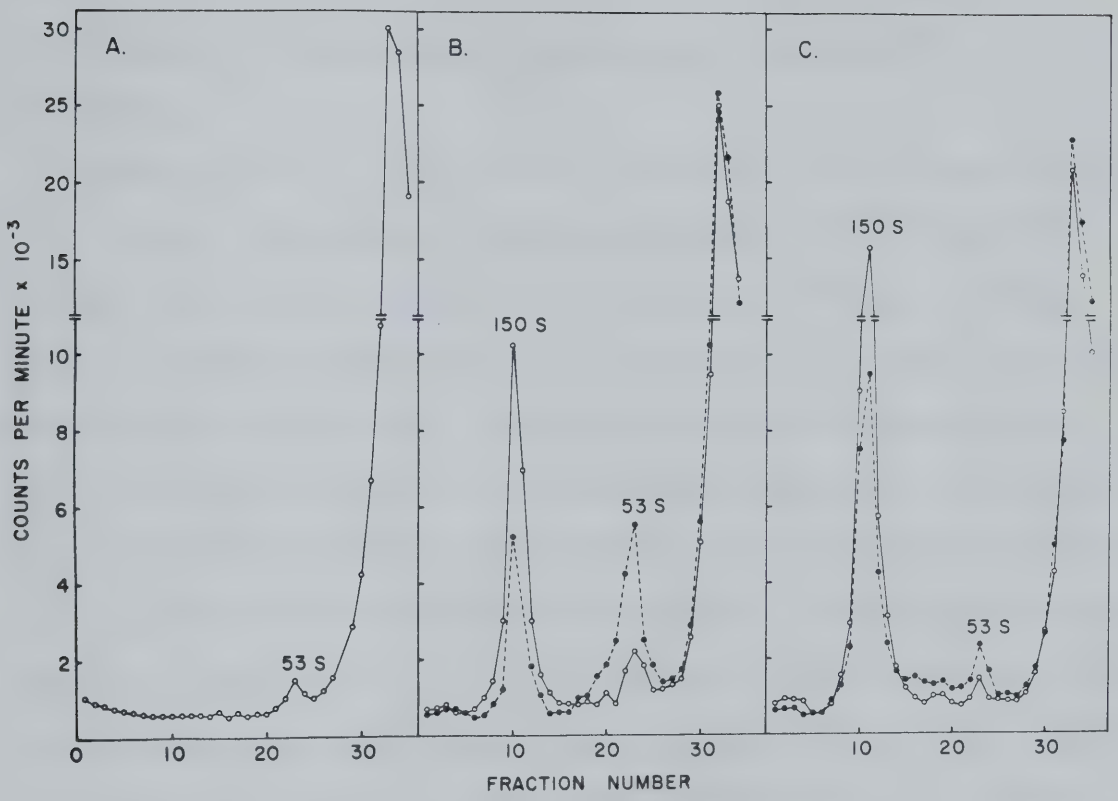


Figure 10. Sedimentation analysis of S_{20} fractions prepared from Mengo virus-infected cells pulse-labeled for 20 min with [^{3}H] amino acids at 4.5 hr postinfection. Centrifugation through 15-45% sucrose gradients was for 13.5 hr at 20,000 rpm (Beckman SW 27.1 rotor). (A) S_{20} fraction prepared immediately after the labeling period. (B) S_{20} fractions prepared after a chase period of 45 min in (○) BME-5% HS and (●) BME-5% HS containing 200 µg of cordycepin/ml. (C) S_{20} fractions prepared after (○) a chase period of 135 min in BME-5% HS and (●) a chase period of 45 min in BME-5% HS containing 200 µg of cordycepin/ml followed by 90 min in BME-5% HS after removal of the cordycepin.

obtained with the S_{20} fraction from cells that were incubated for 135 min in the presence of cordycepin did not differ significantly from that shown in Fig. 10B. The size of the virus peak remained unchanged, while the size of the 53S peak increased only slightly.

Effect of KCl Concentration on Recovery of Virus-Specific Particles

During the course of this study it was found that the concentration of KCl employed has a marked effect on the release of virus-specific particles into the S_{20} fraction and thus on their recovery in the P_{45} pellet. Infected cultures were pulse labeled with [^{3}H] amino acids for 20 min at 5 hr postinfection, after which they were incubated for an additional 75 min in BME-5% HS containing cordycepin (200 µg/ml) before being harvested and homogenized as outlined under Materials and Methods. The homogenate was then divided into six aliquots, the KCl concentration therein was adjusted to 0, 20, 40, 60, 80, and 100 mM, respectively, and the P_{45} fractions were isolated from each as described earlier. Sucrose density gradient analyses gave the results shown in Fig. 11. The concentration of KCl in the suspending buffer appears to have little or no effect on the amount of 14S material present in the P_{45} fraction but does have a rather profound effect on the recovery of both virions and 53S particles. Only trace amounts of both were obtained when the KCl concentration in the cell homogenate was 60 mM or less. Significant quantities of both were recovered in the presence of 80 mM KCl, and the maximum recovery was obtained at 100 mM KCl (higher concentrations of the salt did not increase the yield further).

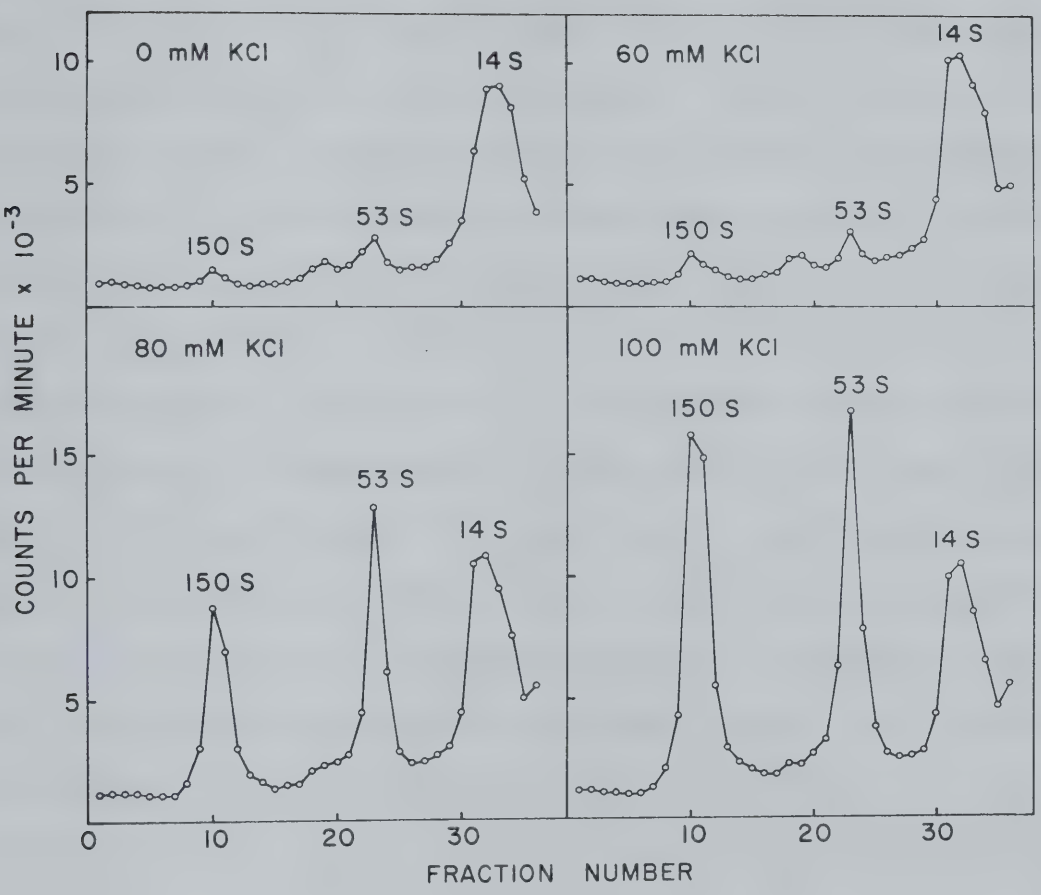


Figure 11. Effect of KCl concentration on the recovery of virus-specific particles from Mengo virus-infected cells. Infected cells were pulse labeled for 20 min with [^{3}H] amino acids at 5 hr postinfection, chased for 75 min in the presence of cordycepin, and then homogenized in homogenizing buffer. The homogenate was then divided into six aliquots in which the KCl concentration was adjusted to 0 (<1), 20, 40, 60, 80, and 100 mM, respectively before P_{45} fractions were isolated. The P_{45} fractions were suspended in TBS and analyzed in 15-45% sucrose gradients (in TBS). Centrifugation was at 20,000 rpm for 13.5 hr (Beckman SW 27.1 rotor).

Effect of Salts (KCl and CsCl) on the Sedimentation Behavior of 53S Particles

When the KCl concentration in homogenates of cells which had been pulse labeled and incubated in the presence of cordycepin as outlined in the preceding section was increased from 100 mM to 150 mM or higher prior to the separation of the P_{45} fraction, the sedimentation profile obtained with that fraction was found to be altered. This finding is illustrated in Fig. 12. The data suggest that when the P_{45} fraction is sedimented from solutions containing a KCl concentration of 150 mM or higher, some of the 53S particles are converted to a more rapidly sedimenting species, the fraction so converted being dependent on the KCl concentration. A similar shift in sedimentation behavior was observed with 53S particles that had been centrifuged to equilibrium in a CsCl gradient (see Fig. 7). When such particles were recovered from the CsCl gradient, dialyzed extensively against TBS and re-examined in a 15-45% sucrose density gradient, they were found to band in a position very close to that occupied by marker R17 (78S) virions (Fig. 13). The sedimentation coefficient of these new particles was estimated to be 75S.

Determination of the Molecular Weights of the 14S, 53S and 75S Particles

The data obtained from pulse-chase experiments, and illustrated in Figs. 8 and 10, suggested not only that the 53S particles is an intermediate in the assembly of Mengo virions, but that the 14S particle is the immediate precursor of the 53S particle. It became important then, to establish the stoichiometric relationship between the two particles, - i.e. to determine the number of 14S particles required to

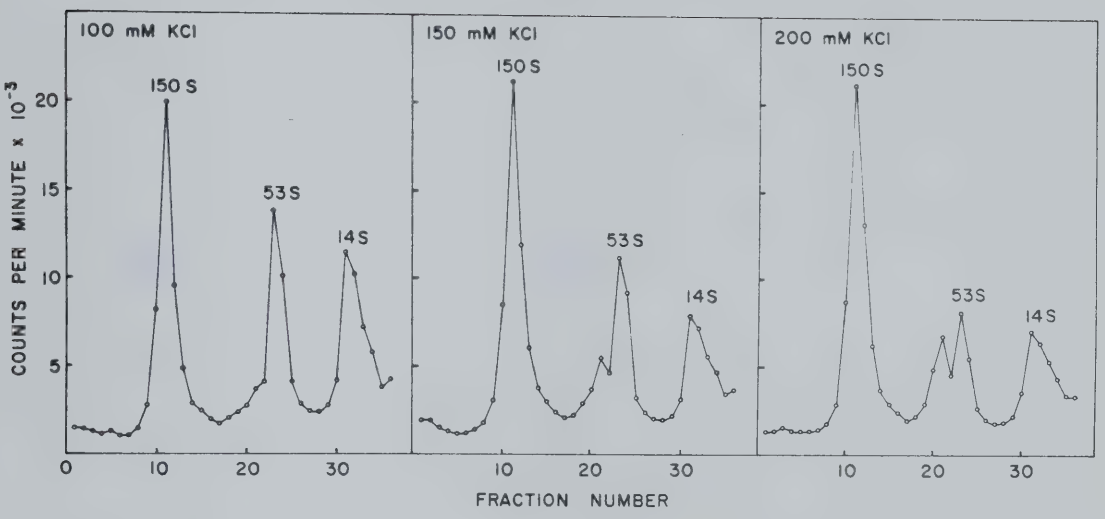


Figure 12. Effect of KCl concentration on the sedimentation behavior of 53S particles. The protocol followed was similar to that discribed in the legend to Fig. 11 except the homogenate was divided into three aliquots in which the KCl concentration was adjusted to 100, 150 and 200 mM respectively before the P_{45} fractions were isolated.

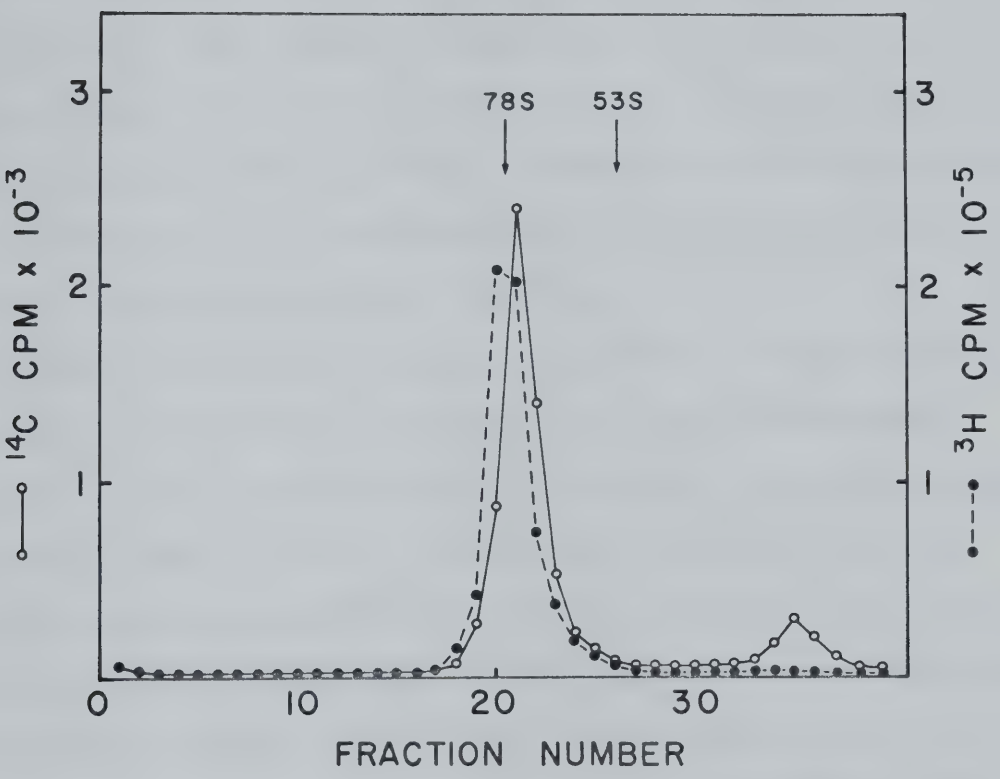


Figure 13. Effect of CsCl on the sedimentation behavior of 53S particles. ^{14}C-amino acid-labeled 53S particles recovered from a CsCl gradient comparable to that illustrated in Fig. 7 were dialyzed for 48 hr against TBS at 4°C and then re-examined in a 16 ml 15-45% sucrose density gradient. Centrifugation was for 13.5 hr at 20,000 rpm (Beckman 27.1 rotor). ^{3}H-uridine-labeled R17 phage (78S) was also present in the same gradient as a marker. (○) ^{14}C counts per minute; (●) ^{3}H counts per minute. The position of 53S particles (before CsCl treatment) in a parallel gradient is also indicated.

form a 53S particle. The conversion of 53S particles to particles having a sedimentation coefficient of 75S, by banding in CsCl or exposure to elevated concentrations of KCl during the isolation procedure, could be due either to some sort of aggregation phenomenon or to a marked conformational change from what would be a highly asymmetric 53S particle to a more compact 75S particle. Since answers to both questions appeared to be important with respect to gaining some understanding of the mechanism of virion assembly, and since both questions could be answered only by determining the molecular weights of all three particles, attempts to do so were undertaken.

It has been shown by Siegel and Monty (1966) that the elution position of a macromolecule subjected to exclusion chromatography through a column of Sephadex G-200 is a function of its Stokes radius, and that the Stokes radius of a macromolecule can be determined by measuring its elution behavior from a column calibrated with macromolecules of known Stokes radii. If both the sedimentation coefficient and the partial specific volume of the macromolecule are also known, an accurate determination of its molecular weight can be made by means of Equation 3 (see Materials and Methods).

Considering the size of the subviral particles involved, a Sepharose 4B column was employed. Such a column was calibrated with the following markers of known Stokes radii (in brackets): Mengo virion (145 Å), R17 bacteriophage (139 Å), fibrinogen (107 Å) and the 13.4S subunit derived from Mengo virus (76 Å). The elution positions of these markers, as well as the elution patterns obtained with the 14S, 53S, and 75S particles, are illustrated in Fig. 14. The void volume (V_o) and the total bed volume

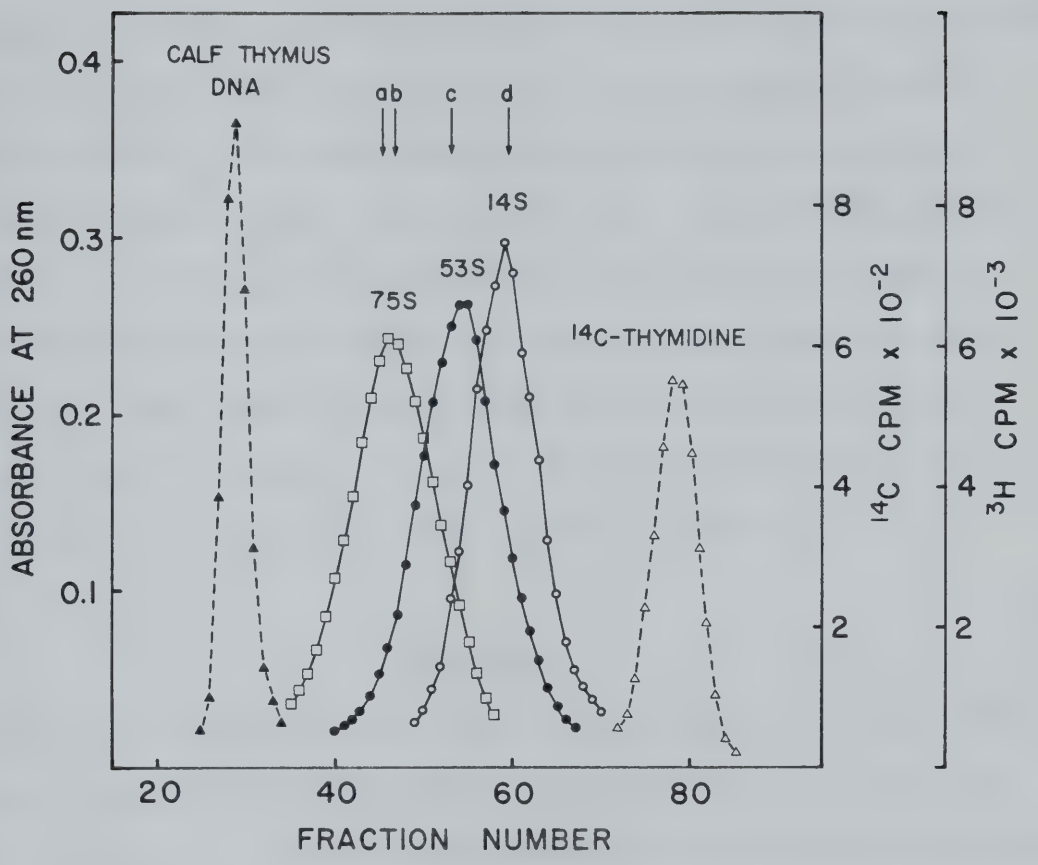


Figure 14. Gel exclusion chromatography of 14S, 53S and 75S particles. Samples (0.8 ml) containing 4 OD_{260} units of calf thymus DNA (for determination of void volume, V_o), approximately 10,000 cpm of ^{14}C-thymidine (for determination of the bed volume of the gel, V_t), and ^{3}H-amino acid-labeled 14S, 53S or 75S peak fractions from sucrose density gradients were applied to a 80cm x 1cm column of Sepharose 4B equilibrated with TBS at 4°C. The components of the mixture were then eluted from the column and the elution profiles were determined as outlined under Materials and Methods. The arrows indicate the elution positions of markers of known Stokes radii: a = Mengo virion; b = R17 bacteriophage; c = fibrinogen; and d = Mengo 13.4S subunit.

(V_t) of the column are indicated by the elution positions of calf thymus DNA and [^{14}C] thymidine respectively. The calibration curve of the column, obtained by plotting $(-\log Kav)^{1/2}$ vs Stokes radii of the markers, is shown in Fig. 15. Having determined the Kav values for the 14S, 53S and 75S particles from their elution positions from the calibrated column, the Stokes radii of all three particles were read from the calibration curve. Knowing the Stokes radii, sedimentation coefficients and partial specific volumes, the molecular weights of the particles were calculated from equation 3. The results are shown in Table 3. They suggest strongly that the 53S particle is composed of five of the 14S subunits [i.e. that it may be represented by $(\varepsilon\alpha\gamma)_{25}$], and that the 75S particle is a dimer of the 53S particle.

Discussion

The results presented herein show clearly that two subviral particles can be isolated from Mengo virus-infected cells: a 14S particle which corresponds to the 14S particle found in cells infected with poliovirus (Phillips *et al.*, 1968), EMC virus (McGregor *et al.*, 1975), and a human rhinovirus (McGregor and Rueckert, 1977) and a 53S particle which has not been described previously. Both particles contain equimolar amounts of the polypeptides ε, α and γ, which correspond precisely to the composition of the previously described 14S particles and to that of the 73S poliovirus empty capsids (Maizel *et al.*, 1967; Jacobson and Baltimore, 1968b; Phillips and Fennell, 1973) and of the 125S provirion described by Fernandez-Tomas and Baltimore (1973).

In considering the morphogenesis of picornaviruses as a group, the one common denominator is the 14S particle, which, it seems clear,

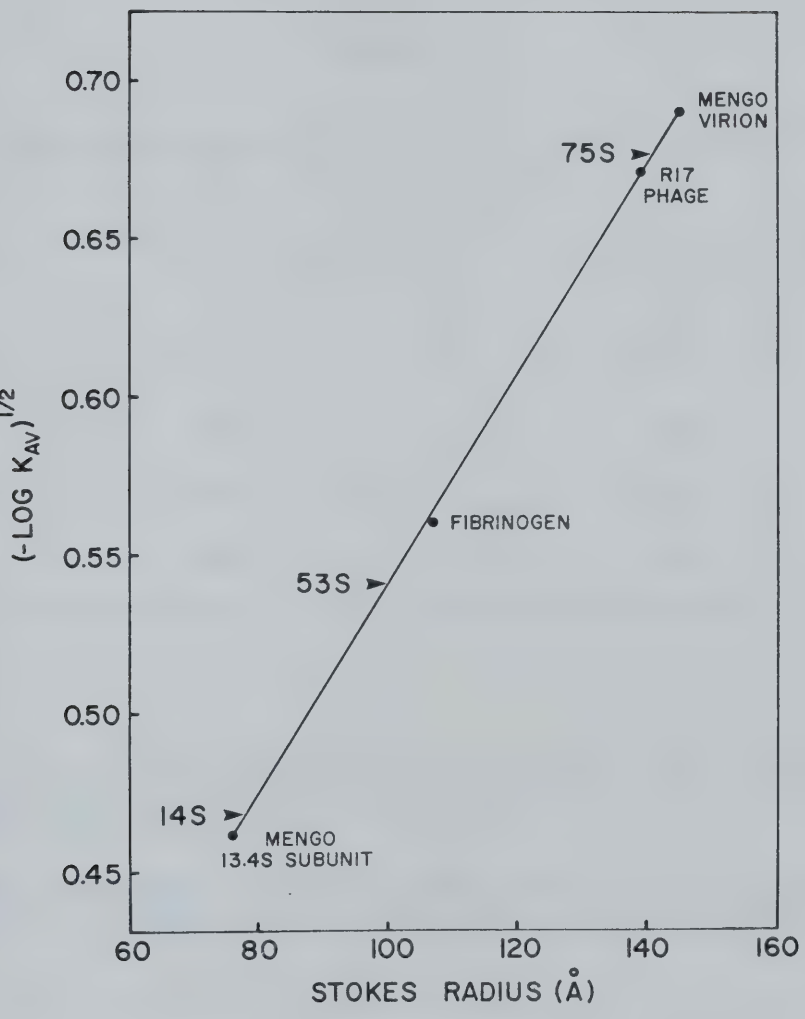


Figure 15. Stokes radii of 14S, 53S and 75S particles as determined from their elution positions [in terms of (-log Kav)$^{\frac{1}{2}}$] on a Sepharose 4B column. The Stokes radii of the markers were determined as outlined in the Materials and Methods section.

TABLE 3

Molecular Weights of 14S, 53S and 75S Particles

	Stokes radius[a] (Å)	Molecular weight[b] ($\times 10^6$)
14S	78	0.46
53S	100	2.23
75S	141	4.45

[a] Average of three separate determinations. The range of values for the 14S, 53S and 75S particles were 77Å-79Å, 99Å-102Å and 139Å-143Å respectively.

[b] Calculated from equation 3, using the value (0.73 ml/gm) for partial specific volume determined by Mak *et al*. (1971).

occupies an early position in the assembly pathway of the entero-, cardio-, and rhinoviruses (McGregor and Rueckert, 1977). The precise role of the 73S empty capsids in the poliovirus system is somewhat less secure. Jacobson and Baltimore (1968b) found that 73S capsids accumulate in infected cells in the presence of 3 mM guanidine hydrochloride, and since the labeled protein was found to be chased into mature virions upon removal of the guanidine, they concluded that the 73S particle is a precursor in the assembly process. However, Ghendon *et al.* (1972) reported that 14S rather than 73S particles accumulate in poliovirus-infected MiO cells (from rhesus monkey tonsils) in the presence of guanidine, and that when the guanidine is removed, the accumulated 14S material is chased into virions without the appearance of 73S capsids. These experiments, coupled with the fact that there is no completely convincing evidence that empty capsids are produced in cardiovirus systems, has led to the suggestion that the 73S particles may not be intermediates in the assembly process (for a review, see Casjens and King, 1975).

The results of the pulse-chase experiments described here suggest that the 53S particle may be a true intermediate in the assembly pathway of Mengo virus. Following a brief labeling period, label can be detected in 53S particles before it appears in mature virions, and during a subsequent chase, label flows from both 14 and 53S particles into the virions. The results of those experiments in which viral RNA synthesis was blocked by cordycepin during the chase period are even more suggestive. The observations that 53S particles accumulate under these conditions and that the label chases into virions when the inhibition of RNA synthesis is reversed are very similar to those made by Jacobson

and Baltimore (1968b) with guanidine-inhibited poliovirus-infected cells and lead us to suggest that the 53S particle is indeed a precursor of the mature Mengo virion.

The data obtained from pulse-chase experiments also suggest that the 14S structure is the immediate precursor of the 53S particle, a view substantiated by the observation that 53S particles can be disassembled into 14S structures by prolonged incubation at room temperature. In this respect the 53S particle resembles the 73S particle found in the poliovirus system, since the latter has also been shown to be an aggregate of 14S particles (Phillips, 1969 and 1971). However, it is clear, particularly from the molecular weight data presented here, that the 53S and 73S particles are not analogous structures.

The finding that the 53S particles described here can be converted, presumably by dimerization, to 75S particles is not unique to the Mengo system. A similar observation was made by Su and Taylor (1976), who reported that 45S particles, isolated in buffer containing 10 mM NaCl from bovine enterovirus 1 - infected cells, can be converted to 80S particles by dialysis against buffer containing 150 mM NaCl. The 53S → 75S conversion suggests that the latter particle may be analogous to the 73S particles found in the poliovirus system. At the same time, the estimated molecular weight of the 75S particle indicates that it may be an incomplete viral capsid [corresponding to $(\alpha,\beta,\gamma,\delta)_{50}$ as opposed to $(\alpha,\beta,\gamma,\delta)_{60}$ for a complete capsid], and this in turn casts some doubt on the assumption that the 73S poliovirus particle is, in fact, a complete empty capsid. It is revealing that the literature contains no reports of any serious attempts to determine the precise molecular weight of the 73S particle.

The validity of the conclusions regarding the composition of the

53S and 75S particles, and of the proposed model for the assembly of the Mengo virion (see following section), depends on the validity of the technique of exclusion chromatography on Sepharose 4B as a method for the determination of Stokes radii (and hence for the calculation of molecular weights). Confidence in the method has been engendered by the reproducibility of the results obtained from three separate estimations (±3-4%), the fact that the plot of $(-\log K_{av})^{1/2}$ vs Stokes radius for the marker particles is strictly linear, and the very close agreement between the molecular weight (4.6×10^5) of the 14S particle estimated by this method and the value of 4.72×10^5 calculated from its composition, $(\varepsilon\alpha\gamma)_5$ (McGregor *et al.*, 1975), and the molecular weights of the individual polypeptides (Ziola and Scraba, 1974).

A proposed scheme for the assembly of Mengo virions, based on the data presented in this chapter, is shown in Fig. 16A. It envisions that five 14S structure units aggregate to form 53S particles, which in turn dimerize to form 75S particles (incomplete capsids). After insertion of the viral RNA into the 75S structure, the virion is completed by the addition of two more 14S structure units and the final, morphogenic cleavage of most of the ε polypeptides.

There is some evidence, albeit circumstantial, that there may be something unique about two of the 14S structure units that comprise the capsids of picornaviruses. Dunker and Rueckert (1971) reported that two of the pentameric subunits produced by the pH dissociation of ME virus are distinguished from the other ten by their insolubility in the dissociation buffer and the presence therein of an uncleaved ε polypeptide. McGregor and Rueckert (1977) found that rhinovirus

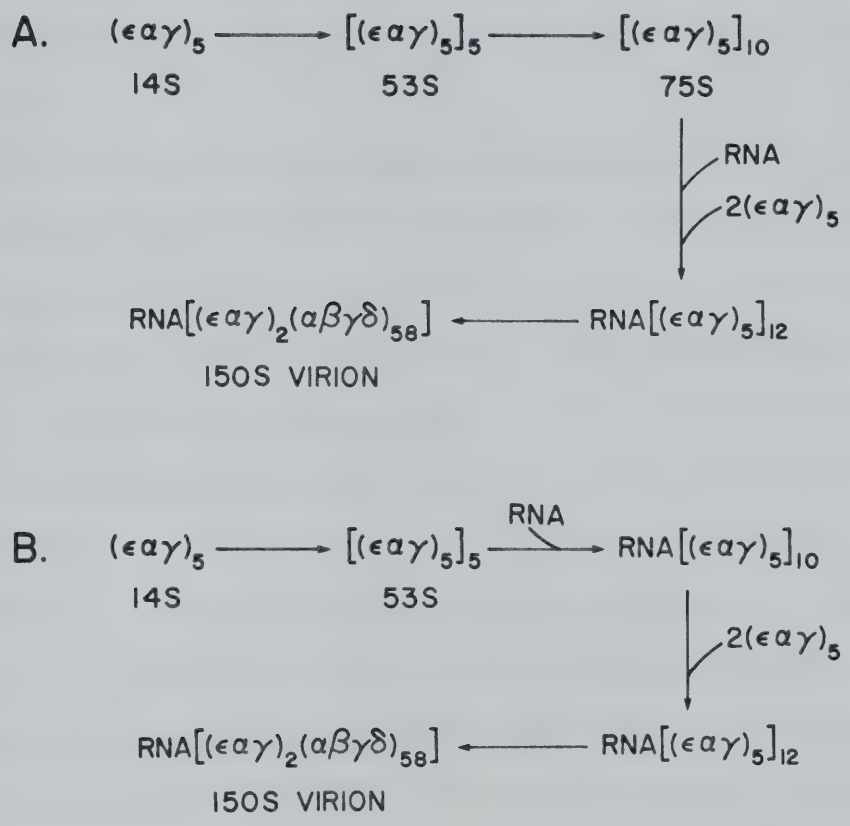


Figure 16. Proposed alternate pathways of Mengo virus morphogenesis.

1A contains approximately ten ε polypeptides per virion, the number that would be present in two 14S structure units. In the light of the assembly scheme proposed here, it is tempting to speculate that the uncleaved ε residues found in a number of picornaviruses arise from the two 14S structure units that complete the encapsidation of the viral genome.

An obvious criticism of the assembly scheme shown in Fig. 16A is that while stable 75S particles can be formed readily *in vitro* from 53S particles, they have not been detected in extracts of infected cells as prepared in these investigations. One can rationalize the failure to find 75S particles on the grounds that the assembly of virions is a highly concerted process, and that, as a result, the short-lived 75S species may not accumulate to levels that make its detection possible. Be that as it may, the 75S particle described here (i.e. an incomplete viral capsid) is a more credible putative intermediate in the assembly process than is a complete empty capsid (the 73S particle of the poliovirus system). Certainly it is easier to envision how a viral genome could be packaged (inserted) into an incomplete capsid than into a complete one.

An alternative assembly scheme - also compatible with the experimental evidence and shown in Fig. 16B - is one in which the 75S particle is not formed, but in which two 53S particles interact with a molecule of viral RNA to form an intermediate described by $RNA[(\varepsilon\alpha\gamma)_5]_{10}$. The insertion of two additional 14S particles and the cleavage of ε residues to β and δ polypeptides would then complete the assembly process.

Available evidence suggests that at least in the poliovirus system, viral RNA replication and particle formation are coupled processes that

occur in association with the smooth cytoplasmic membranes (Caliguiri and Compans, 1973). It thus seems likely that in the Mengo virus system, 14S precursor particles aggregate on these membranes to form 53S particles, which in turn interact with newly synthesized viral RNA via one of the two pathways shown in Fig. 16. The observation that the release of 53S particles and of newly assembled virions (but not of 14S particles) into S_{20} fraction is strongly dependent on the KCl concentration of the buffer in which the manipulations are carried out is compatible with this hypothesis.

CHAPTER IV

STUDIES OF TWO TEMPERATURE-SENSITIVE MUTANTS OF MENGO VIRUS

Introduction

Current genetic studies with animal viruses are based in large measure on the use of temperature sensitive (ts) mutants, and conditional lethal mutants of this class have been used for genetic and biochemical investigations of representative members of a number of groups of mammalian viruses (see for example, Fields and Joklik, 1969; Toyoshima and Vogt, 1969; Holloway *et al.*, 1970; Portner *et al.*, 1974; Ghendon *et al.*, 1975; Strauss *et al.*, 1976). Within the picornavirus group, ts mutants have been used for the genetic analysis of poliovirus (Cooper, 1969) and foot and mouth disease (FMD) virus (Lake *et al.*, 1975; McCahon *et al.*, 1977), and reports of the isolation and partial characterization of ts mutants of members of the cardiovirus subgroup have come from this (Downer *et al.*,1976) and other (Bond and Swim, 1975; Radloff, 1978) laboratories.

One of the main objectives of studies with ts mutants is to define the functions of viral gene products, particularly the non-structural polypeptides, which, for the cardioviruses, have been identified and designated G, H, F, I and E. Although there is circumstantial evidence that polypeptide E may be the viral coded component of the virus-specific RNA replicase (Rosenberg *et al.*, 1972; Traub *et al.*, 1976), the biological roles of the others remain unknown.

Of the 18 ts mutants of Mengo virus isolated in this laboratory, 11 and 7 have been shown to be RNA^- and RNA^+ respectively, and 5 have

been found to have significantly lower thermal stabilities than the wild-type virus, suggesting that they have a ts defect in one or more of the structural polypeptides. Two of this group of 5 are RNA^- mutants, which suggests that they may be double mutants.

One such mutant, ts 135, chosen for its high 33°/39° plaque ratio and its ability to grow to high titer at the permissive temperature, has been studied in some detail, the objectives being to determine 1) whether its RNA^- phenotype reflects a ts lesion in the viral RNA replicase or a defect in the synthesis of the viral coded component thereof, and 2) whether its low thermal stability (half life at 39° = 70 min, as compared to 600 min for the wt virus) can be accounted for by a detectable defect in one or more of its structural polypeptides or their precursors. The data presented in this chapter show that the viral RNA replicase produced in cells infected with mutant ts 135 is temperature sensitive, and that the normal cleavage of precursors of the structural polypeptides of the virion is partially blocked at the non-permissive temperature.

For purposes of comparison, another RNA^- (but relatively thermostable) mutant, ts 520, was also studied. The results show that although this mutant, like ts 135, is temperature sensitive with respect to RNA replication, the cleavage of its structural polypeptide precursors is normal at the non-permissive temperature.

Other studies of the two ts mutants have also provided supporting evidence for the proposal that the 53S particle described in Chapter III is a normal intermediate in the assembly of Mengo virions.

Materials and Methods

Virus

The wild-type virus used was the plaque variant of Mengo virus designated M-Mengo (Ellem and Colter, 1961). The isolation of the mutants, ts 135 and ts 520, from a mutagenized (nitrous acid) stock of M-Mengo, and their partial characterization have been described previously (Downer *et al.*, 1976).

Infection of Monolayers and Labeling of Viral Ribonucleates and Polypeptides

Cells grown in petri dishes (60 x 15 mm) were infected at either 33° (permissive temperature) or 39° (non-permissive temperature) at an estimated multiplicity of 100 PFU/cell with either wt or ts Mengo virus suspended in virus diluent. After incubation for 1 hr, the monolayers were washed with warm BME-5% HS and then incubated at 33° or 39° in the same medium.

To determine growth curves and to follow the synthesis of viral ribonucleates in temperature-shift experiments, the medium on replicate monolayers was replaced at 2 hr post-infection with BME-5% HS containing [^{3}H] uridine (10 µCi/ml) and actinomycin D (3 µg/ml). Cultures were harvested at intervals and the cells disrupted by sonic vibration. Virus production was determined by plaque assay at 33° and viral RNA synthesis was monitored by applying aliquots of the cell lysates onto filter paper disks (Whatman No. 3, 2.3 cm diameter) which were air-dried, washed sequentially with 10% TCA, 5% TCA, ethanol and acetone before being placed in scintillation vials. Radioactivity was measured in the presence of 10 ml of Aquasol scintillation fluid (New England Nuclear, Corp.) in a Beckman liquid scintillation spectrometer

(model LS-230).

Viral polypeptides were labeled by incubating infected cells for 15 min in amino-acid deficient Eagle's medium containing 1% HS and [^{3}H] amino acids (50 µCi/ml). Labeling was done at 4.5 hr postinfection when cells were incubated at 39° and at 7.5 hr when cells were incubated at 33°. In both cases the cultures were incubated in amino acid-deficient medium for the hour immediately preceding the labeling period. When labeling was followed by a chase period, the monolayers - after removal of the radioactive medium - were washed once with Dulbecco's phosphate-buffered saline (PBS) and incubated for the desired period of time in BME-5% HS.

Extraction and Analysis of Viral RNA

Infected monolayers that had been incubated with [^{3}H] uridine were scraped off the petri dishes and resuspended at a concentration of 1.5×10^6 cells/ml in a buffer containing 0.1 M NaCl, 1 mM EDTA, 0.01M Tris-HCl, pH 7.4 (TNE). After the addition of SDS and carrier yeast tRNA to final concentrations of 1% and 2 mg/ml respectively, RNA was extracted by shaking for 10 min at room temperature with an equal volume of redistilled phenol saturated with TNE. After low speed centrifugation, the phenol phase was removed and the aqueous phase was extracted twice more with phenol. RNA was precipitated by adding 2.5 volumes of ethanol to the aqueous phase and incubating the mixture overnight at -20°. The precipitate was then dissolved in DNase buffer (0.1 M NaCl, 1 mM $MgCl_2$, 0.01 M Tris-HCl, pH 7.4) to which RNase-free DNase was added to a concentration of 20 µg/ml. After 30 min at room temperature, the solution was made 10 mM in EDTA, extracted with TNE-saturated phenol, and the RNA was again precipitated with ethanol.

The RNA was then vacuum-dried and dissolved in 0.2 ml TNE containing 0.5% SDS, 10% sucrose and 0.002% bromophenol blue.

Electrophoresis of viral RNA was carried out according to the procedure of Noble *et al.* (1969) with minor modifications. Two percent polyacrylamide gels containing 0.5% agarose were cast in 0.6 x 10cm glass tubes coated with dichlorodimethylsilane, and were pre-electrophoresed for 1 hr at 7 mA/gel before RNA samples (50 µl) were applied. Electrophoresis was carried out at 7 mA/gel for 4 hr after which the gels were fractionated using the automatic aliquogel fractionator (Gilson Medical Electronics). The fractions were incubated overnight at 65° in 0.5 ml of 30% H_2O_2 and the radioactivity in each was measured as described earlier.

Preparation of Crude Enzyme Extracts

The procedure employed was essentially the same as that described by Baltimore and Franklin (1963). Monolayer cultures in petri dishes (100 x 15 mm) were harvested 9 hr after infection at 33° and the cells resuspended in distilled water at a concentration of 10^7/ml. After 5 min at 0°, the cells were disrupted in a Dounce homogenizer, and the tonicity of the homogenate was adjusted by the addition of 10x concentrated buffer to give final concentrations of 0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, and 1.5 mM $MgCl_2$ (STM). The nuclei were removed by low speed centrifugation, after which the supernatant was centrifuged for 20 min at 30,000 g (16,000 rpm, JA-20 rotor, Beckman J21 centrifuge) and the pellet obtained was resuspended in STM buffer at 1 mg of protein per ml as determined by the method of Lowry *et al.* (1951).

In vitro RNA Polymerase Assay

This was carried out in a standard reaction mixture consisting of 50 µmoles of Tris-HCl, pH 8.0, 50 µmoles of KCl, 10 µmoles of $MgCl_2$, 2 µg of actinomycin D, 0.1 µmole of dithiothreitol, 0.15 µmole each of ATP, CTP and GTP, 10 µCi of [^{3}H] UTP, and 0.4 ml of enzyme preparation (0.4 mg protein) in a total volume of 1 ml. Incubation was carried out at either 33° or 39° after pre-incubating the enzyme preparation for 30 min at 33° or 39°. At various times of incubation, 100 µl aliquots of the reaction mixture were placed on filter paper disks pre-treated with sodium pyrophosphate. The disks were then washed sequentially with 10% TCA, 5% TCA, ethanol and acetone before the radioactivity contained thereon was measured.

Polypeptide Analysis of Cell Lysates

Lysates were prepared by removing the medium from monolayers that had been pulse-labeled with [^{3}H] amino acids, washing the monolayers three times with cold PBS and adding 0.2 ml of "lysis mixture": 0.01 M sodium phosphate buffer, pH 7.2, containing 2% SDS, 5% β-mercaptoethanol, and 10^{-3}M phenylmethylsulfonyl fluoride (PMSF). Aliquots (100 µl) of the lysates, to which glycerol and bromophenol blue had been added to give final concentrations of 10% and 0.002% respectively, were heated at 100° for 5 min before being layered onto 7.5% polyacrylamide gels. Electrophoresis was carried out as described in the Materials and Methods section of Chapter III.

Preparation of Cell Extracts for *in vitro* Cleavage Studies

Monolayers of ts 135-infected cells were pulse labeled for 15 min with [^{3}H] amino acids (50 µCi/ml) at 7.5 hr postinfection, then harvested immediately and resuspended in cold PBS at a density of 2×10^7 cells/ml. After the cells were ruptured in a Dounce homogenizer,

the nuclei were removed by low speed centrifugation and a cytoplasmic extract containing labeled viral polypeptide precursors was obtained. An unlabeled cytoplasmic extract was prepared at the same time from wt Mengo virus-infected cells.

Sucrose Density Gradient Analyses of Cell Extracts

The preparation of cytoplasmic supernatnats (S_{20}) and the subsequent analysis of virus-related particles by means of sucrose gradient centrifugation has been described in the previous chapter.

Materials and Radioisotopes

Actinomycin D was purchased from Schwarz/Mann, Orangeburg, New York, carrier tRNA from Mann Research Labortories, Orangeburg, New York, deoxyribonuclease I from Worthington Biochemical Corp., Freehold, New Jersey, cycloheximide from Calbiochem, California, dithiothreitol and cordycepin from Sigma Chemical Company, St. Louis, Missouri, ATP, CTP and GTP from Raylo Chemicals Ltd., Edmonton, Alberta. [^{3}H] uridine (24.2 Ci/mmole), [^{3}H] amino acids (1.0 mCi/ml) and [^{3}H] UTP (37.3 Ci/mmole) were obtained from New England Nuclear Corp.

Results

A. Studies of Mutant *ts* 135

Virus Production and Viral RNA Synthesis

The replication of mutant ts 135 at the permissive and non-permissive temperatures is illustrated in Fig. 17. Whereas the growth curve obtained at 33° is essentially identical to that obtained with the wt virus at the same temperature, no replication can be detected when ts 135-infected cells are held at 39° throughout infection. Also shown in Fig. 17 is the effect on virus replication of shifting ts 135-

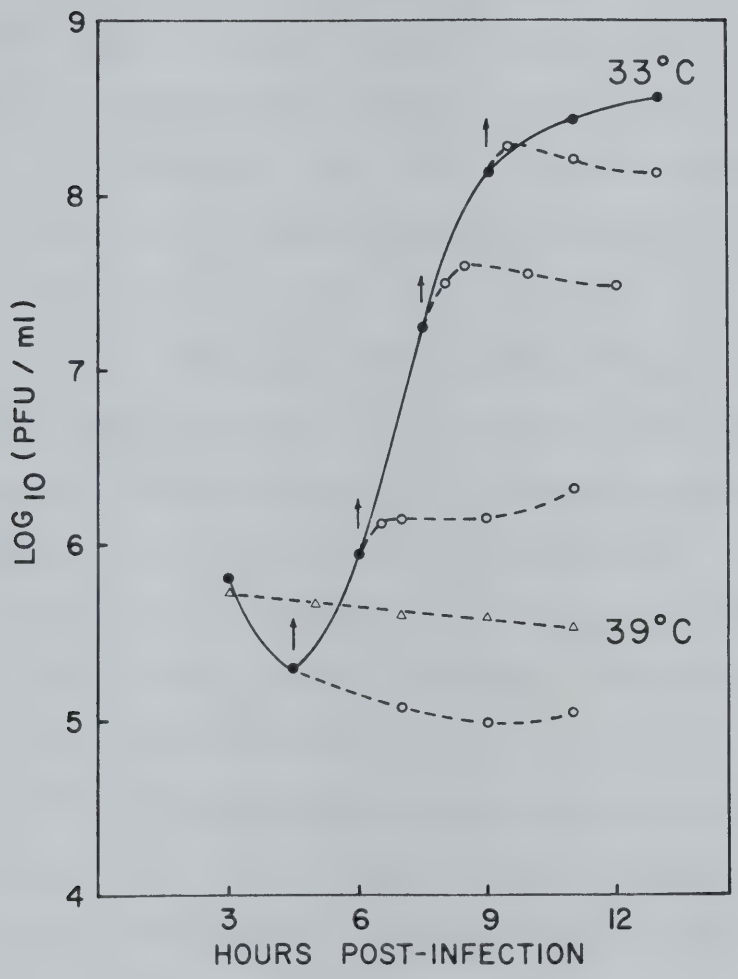


Figure 17. Growth behavior of mutant ts 135 at 33° and 39°. Virus production at 33° (●), at 39° (△), and after temperature shift-up from 33° to 39° (○) at times indicated by arrows.

infected cells from 33° to 39° at various times during the replicative cycle. As may be seen, shifting the cultures from 33° to 39° results in a rapid cessation of virus growth regardless of the time at which the temperature shift-up is made.

The data obtained from a similar experiment, in which the synthesis of viral RNA in ts 135-infected cells was monitored at 33° and 39°, and in cultures that were shifted from 33° to 39° at various times post-infection, are illustrated in Fig. 18. At 39°, [^{3}H] uridine incorporation does not exceed the level observed in uninfected cells incubated in the presence of actinomycin D, while at 33°, viral RNA synthesis is essentially the same as that seen at 33° in cells infected with wt virus at the same input multiplicity. The synthesis of viral RNA in ts 135-infected cells is shut off rapidly when cultures are shifted from 33° to 39°, and the inhibition appears to be irreversible. In cultures shifted to 39° at 6 hr post-infection and held at that temperature for 2 hr, RNA production does not resume when the cultures are returned to the permissive temperature.

To determine whether the synthesis of all species of virus-specific RNA is impaired at 39°, wt- and ts 135-infected cells at mid-logarithmic phase of virus production were either pulse-labeled with [^{3}H] uridine (30 μCi/ml) for 30 min at 33°, or were shifted to 39° for 30 min before being pulse-labeled. RNA was then extracted and analyzed by polyacrylamide gel electrophoresis. The results, illustrated in Fig. 19, show that in ts 135-infected cells the synthesis of all three species of virus-specific RNA - namely, replicative intermediate (RI), replicative form (RF), and single-stranded (ss) RNA - is inhibited to approximately the same extent after temperature shift-up from 33° to

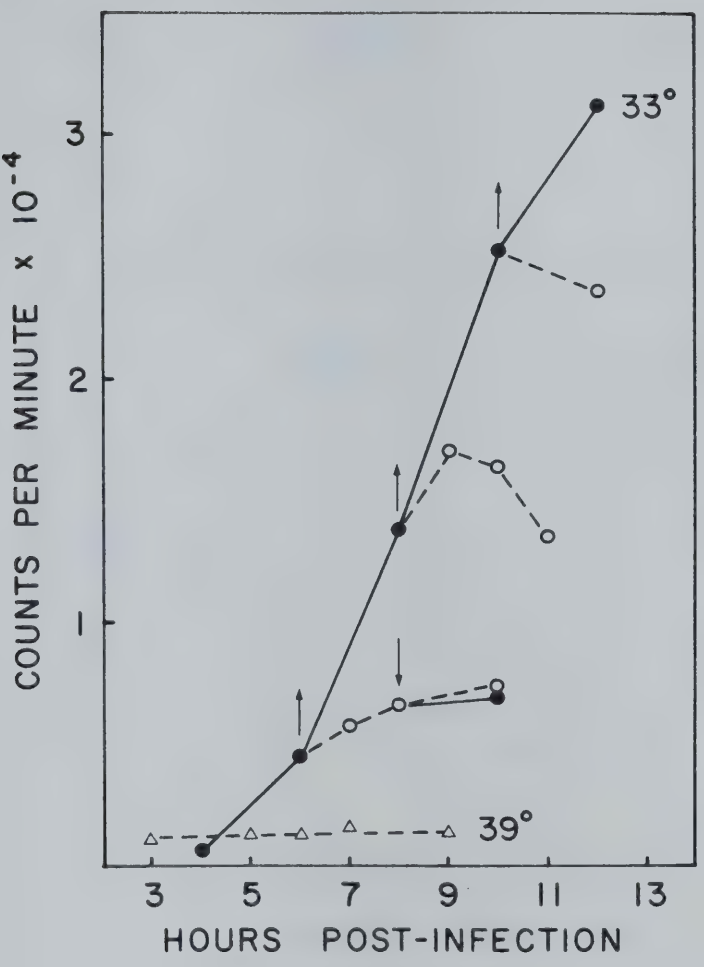


Figure 18. Effect of temperature on the synthesis of ts 135 viral RNA. ^{3}H-uridine (10 μCi/ml) was added to replicate cultures of infected cells at 2 hr postinfection. At various times thereafter, the cultures were harvested, the cells were lysed, and the lysates were analyzed for acid-insoluble radioactivity. (●) RNA synthesis at 33°, (Δ) RNA synthesis at 39°. The arrows indicate the times at which the cultures were shifted from 33° to 39° (↑) or from 39° to 33° (↓): (○) RNA synthesis after shift-up to 39°.

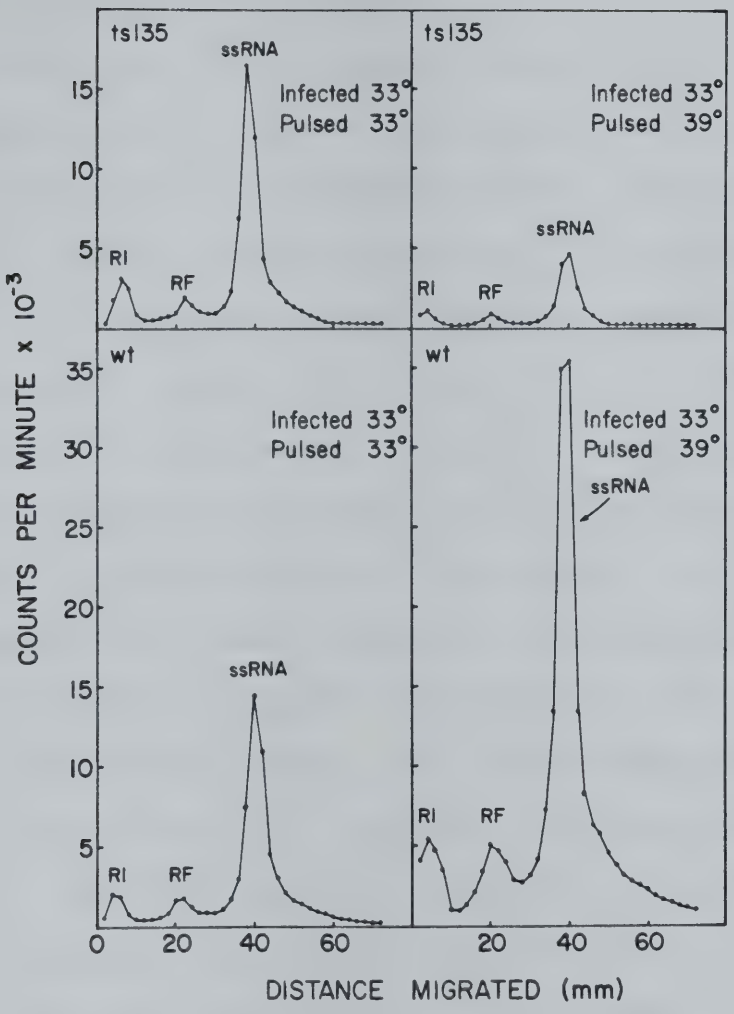


Figure 19. Electrophoresis in SDS-2% polyacrylamide gels of ts 135 and wt virus-specific RNAs synthesized at 33° and 39°. Infected cells at 7 hr postinfection were either pulse-labeled with [^{3}H] uridine (30 μCi/ml) for 30 min at 33°, or were shifted to 39° for 30 min before being pulse-labeled. RNA was then extracted and analyzed as described in Materials and Methods.

39°. In wt virus-infected cells on the other hand, the temperature shift results in an increase in the level of all three species of RNA.

Stability of Viral RNA Polymerase

One, and perhaps the most obvious, explanation for the RNA$^-$ phenotype of mutant ts 135 is that the virus-specific RNA polymerase (replicase) produced in cells infected with the mutant is rapidly inactivated at the non-permissive temperature. This prompted an examination of the *in vivo* stability of the enzyme. Replicate monolayers of both wt- and ts 135-infected cells were set up. At 9 hr post-infection (at 33°), cycloheximide (final conc. = 30 µg/ml) was added to half the cultures in each group. Half of the cultures in each of the 4 groups (ts- and wt-infected; ± cycloheximide) were then shifted to 39°, and at 10 min intervals thereafter duplicate cultures from each group were pulse-labeled for 5 min with [^{3}H] uridine (30 µCi/ml). After the 5 min labeling period, cells were harvested and assayed for acid-insoluble radioactivity. The results are shown in Fig. 20.

At 33°, and in the absence of cycloheximide (Fig. 20A) the rate of viral RNA synthesis increases between 9 and 10 hr post-infection in both wt- and ts 135-infected cells, due presumably to the continued synthesis of the polymerase during that time. At 39° however (Fig. 20C), the rate of RNA synthesis increases in wt-infected cells, while the rate in ts 135-infected cells, after a small initial increase, decreases very rapidly. Since cycloheximide at the concentration employed causes a very rapid and virtually complete inhibition of protein synthesis, measurements of the rate of viral RNA synthesis in cells incubated in the presence of cycloheximide provide an estimate of the stability of the viral RNA polymerase synthesized prior to the addition

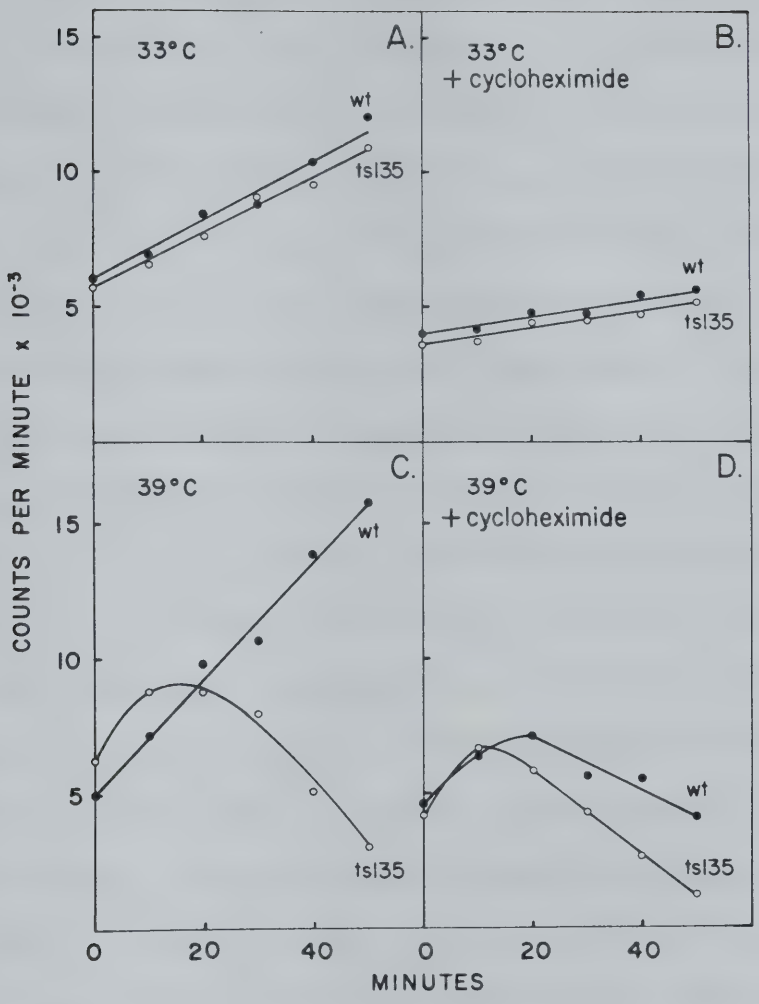


Figure 20. *In vivo* stability of ts 135 and wt viral RNA polymerase. Ts 135 and wt virus-infected cultures were pulse-labeled with ^{3}H-uridine (30 µCi/ml) for 5 min at 10 min intervals between 9 and 10 hr postinfection. (A) cells pulse-labeled at 33°, no cycloheximide added. (B) cells pulse-labeled at 33°, cycloheximide (30 µg/ml) added at 9 hr postinfection. (C) cells pulse-labeled after temperature shift-up at 9 hr postinfection, no cycloheximide added. (D) cells pulse-labeled after temperature shift-up and addition of cycloheximide (30 µg/ml) at 9 hr postinfection. Cultures were harvested immediately after the pulse, the cells were lysed, and the lysates were analyzed for acid-insoluble radioactivity.

of the drug. The data illustrated in Fig. 20B show that, at 33°, the enzyme in both wt- and ts 135-infected cells is stable (for at least one hour). At 39° (Fig. 20D), although both the wt and mutant enzymes are inactivated, the enzyme in ts 135-infected cells is significantly more thermolabile than is the enzyme in wt-infected cells.

The thermal inactivation of the viral replicase can also be demonstrated *in vitro*. Crude enzyme extracts prepared from both wt- and ts 135-infected cells at mid-logarithmic phase of virus production at 33° were assayed for RNA polymerase activity at 33° and 39° by measuring the incorporation of [^{3}H] UTP in a cell-free system. As illustrated in Fig. 21, the two preparations have comparable activity when assayed at 33° after a preincubation period of 30 min at 33°. On the other hand, while preincubation at 39° for 30 min leads to some loss in the activity of the viral RNA polymerase from wt-infected cells when assayed at 39°, it causes an almost complete inhibition of the enzyme from ts 135-infected cells, whether the assay is carried out at either 33° or 39°. These data are consistent with those obtained from studies of the *in vivo* stability of the ts 135-specific RNA polymerase, and show not only that the enzyme itself is thermolabile, but that the inactivation at 39° is irreversible.

Synthesis of Virus-Specific Polypeptides

The synthesis of virus-specific polypeptides in cells infected with mutant ts 135 was investigated by the method of SDS-polyacrylamide gel electrophoresis. In these experiments, cells were pulse-labeled with a mixture of [^{3}H] amino acids at 7.5 hr post-infection, a time which (at 33°) corresponds to the mid-logarithmic phase of virus production. By 7.5 hr post-infection, cellular protein synthesis is almost completely

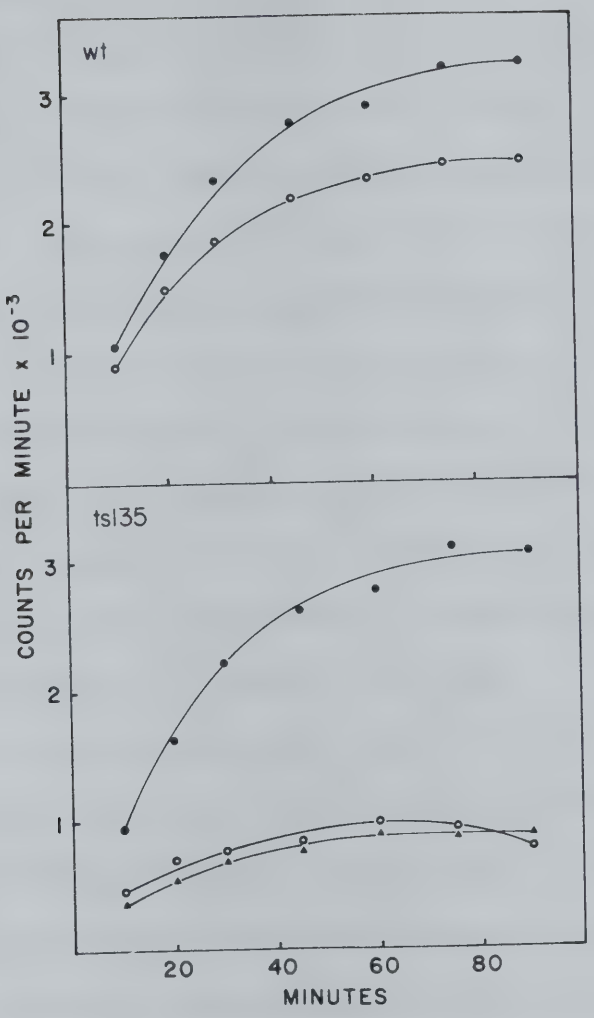


Figure 21. *In vitro* stability of ts 135 and wt viral RNA polymerase. The preparation of crude enzyme extracts from ts 135 and wt virus-infected cells at 9 hr postinfection, and the conditions employed for *in vitro* RNA polymerase assays were as described in Materials and Methods. (●) assayed at 33° after preincubating the enzyme preparation for 30 min at 33°, (○) assayed at 39° after preincubating the enzyme preparation for 30 min at 39°, (▲) assayed at 33° after preincubating the enzyme preparation for 30 min at 39°.

shut off, so the virus-specific polypeptides may be identified readily.

Illustrative electropherograms are shown in Fig. 22. The upper panel shows the characteristic pattern obtained with a lysate of ts 135-infected cells prepared immediately after a 15 min labeling period at 33°. The pattern obtained with a lysate of cells, pulse-labeled for 15 min at 33° and then incubated for an additional 90 min at the same temperature in the absence of labeled amino acids is shown in the lower panel. Both patterns are essentially identical to those obtained with lysates of wt virus-infected cells prepared under the same conditions, and illustrate the fact that with Mengo (as with all picornaviruses) the capsid and noncapsid virus proteins are produced by cleavage of large precursor proteins. Polypeptides A and B are precursors of the structural polypeptides α,β,γ and δ, while C and D are precursors of polypeptide E (Paucha *et al.*, 1974).

When ts 135-infected cells were shifted to 39° at 7 hr post infection and held at that temperature for 30 min before being pulse-labeled - or pulse-labeled and then incubated for an additional 90 min at 39° - a somewhat different result was obtained. As may be seen from Fig. 23, the pattern obtained with a lysate prepared immediately after a 15 min labeling period at 39° (upper panel) is identical to that obtained with a lysate prepared immediately after pulse labeling at 33°. However, the pattern obtained with a lysate prepared after a chase period of 90 min at 39° differs from the one shown in the lower panel of Fig. 22. Firstly, substantial amounts of the structural polypeptide precursors A and B remain after a chase period of 90 min at 39°, suggesting that the cleavage of these proteins is partially blocked at this temperature. Secondly, although small amounts of α

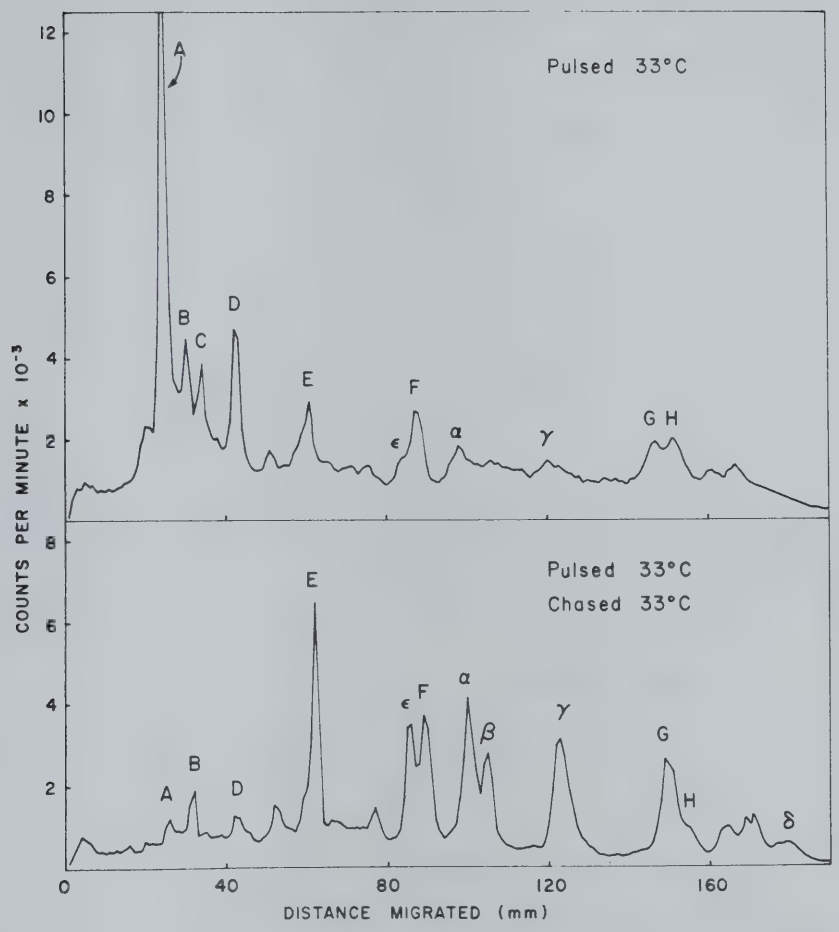


Figure 22. Electrophoresis in SDS - 7.5% polyacrylamide gels of lysates of ts 135-infected cells, pulse-labeled for 15 min with ^{3}H-amino acids (50 μCi/ml) at 7.5 hr postinfection at 33°. Upper panel: cells were lysed immediately after the labeling period. Lower panel: cells were lysed after a chase period of 90 min.

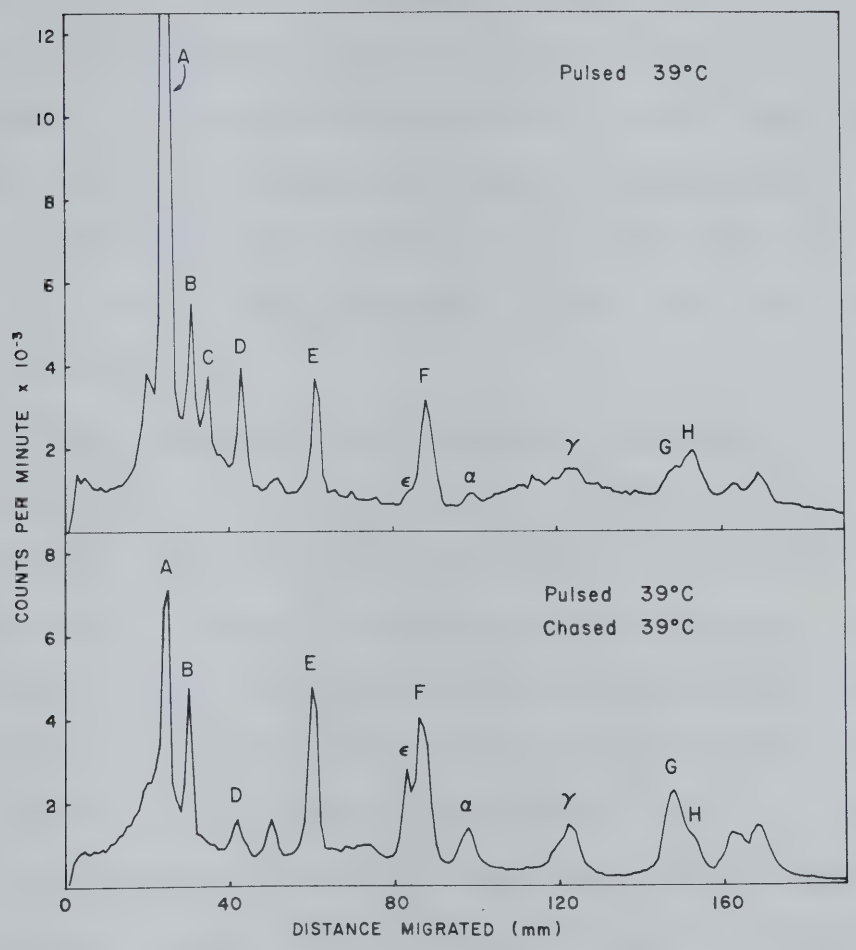


Figure 23. Electrophoresis in SDS-7.5% polyacrylamide gels of lysates of ts 135-infected cells, pulse-labeled at 39° for 15 min with [^{3}H] amino acids (50 μCi/ml) after the cells were shifted at 7 hr post-infection from 33° to 39° for 30 min. Upper panel: cells were lysed immediately after the labeling period. Lower panel: cells were lysed after a chase period of 90 min at 39°.

and γ are produced during a 90 min chase at 39°, the lysate contains not a trace of polypeptides β and δ. The complete absence of β and δ is explained by the fact that the cleavage of ε → β + δ takes place during the final stages of assembly of progeny virions. That being the case, one would not expect to see that cleavage at the non-permissive temperature in cells infected with an RNA^- mutant. These cleavage defects appear to be irreversible since no additional loss of radioactivity from polypeptides A, B or ε was detected in lysates of cells which, after being pulse-labeled and chased at 39°, were shifted back to 33° for 60 min.

In vitro Cleavage of Viral Polypeptide Precursors

The cleavage defect could reflect a temperature sensitive mutation in either the putative virus-coded protease, believed to be responsible for secondary cleavages of capsid polypeptide precursors, or in the precursor(s) itself. The latter mutation would presumably give rise to a protein whose conformation would be altered at 39° in such a way as to inhibit its cleavage by the appropriate protease. In an attempt to distinguish between these two possibilities, the *in vitro* cleavage of precursor proteins A and B synthesized in ts 135-infected cells was examined.

A cytoplasmic extract was prepared from ts 135-infected cells that had been pulse-labeled for 15 min with [^{3}H] amino acids after incubation for 7.5 hr at 33°. Aliquots of the extract, which contained the labeled precursor proteins and the cleavage enzyme(s), were then incubated for 2 hr 1) at 33°, 2) at 39° and 3) at 39° in the presence of 2 volumes of an unlabeled extract prepared from wt virus-infected cells, after which all samples were analyzed for labeled viral polypeptides

by SDS-polyacrylamide gel electrophoresis. The results are presented in Fig. 24.

The labeled polypeptides of the cytoplasmic extract prepared from ts 135-infected cells immediately after the pulse are shown in Fig. 24A. A two hour incubation at 33° resulted in an almost complete disappearance of the large polypeptide precursors A and B (Fig. 24B). However, incubation of the extract at 39° did not bring about a comparable reduction in the amounts of proteins A and B (Fig. 24C). Moreover, the addition of unlabeled extract prepared from wt virus-infected cells to the labeled extract during incubation at 39° did not enhance the cleavage of the two precursors to any significant extent (Fig. 24D). These data thus suggest that the cleavage defect exhibited by ts 135 at 39° reflects a temperature sensitive mutation in the structural polypeptide precursor A (and B).

Synthesis of *wt* Viral Polypeptides in the Presence of Cordycepin

Additional evidence that the cleavage defect exhibited by mutant ts 135 is the result of a second temperature sensitive mutation, rather than a secondary effect of the inhibition of RNA synthesis at 39°, was obtained from a study of the cleavage pattern in wt virus-infected cells in the presence of cordycepin, an inhibitor of viral RNA synthesis. The results are illustrated in Fig. 25, from which it is clear that although the cleavage of ε to β + δ is blocked (as expected), the cleavage of the large precursor molecules to stable, viral gene products occurs normally in the absence of viral RNA synthesis.

B. Studies of Mutant *ts* 520

Virus Production and Viral RNA Synthesis

The replication of mutant ts 520 and the synthesis of viral RNA

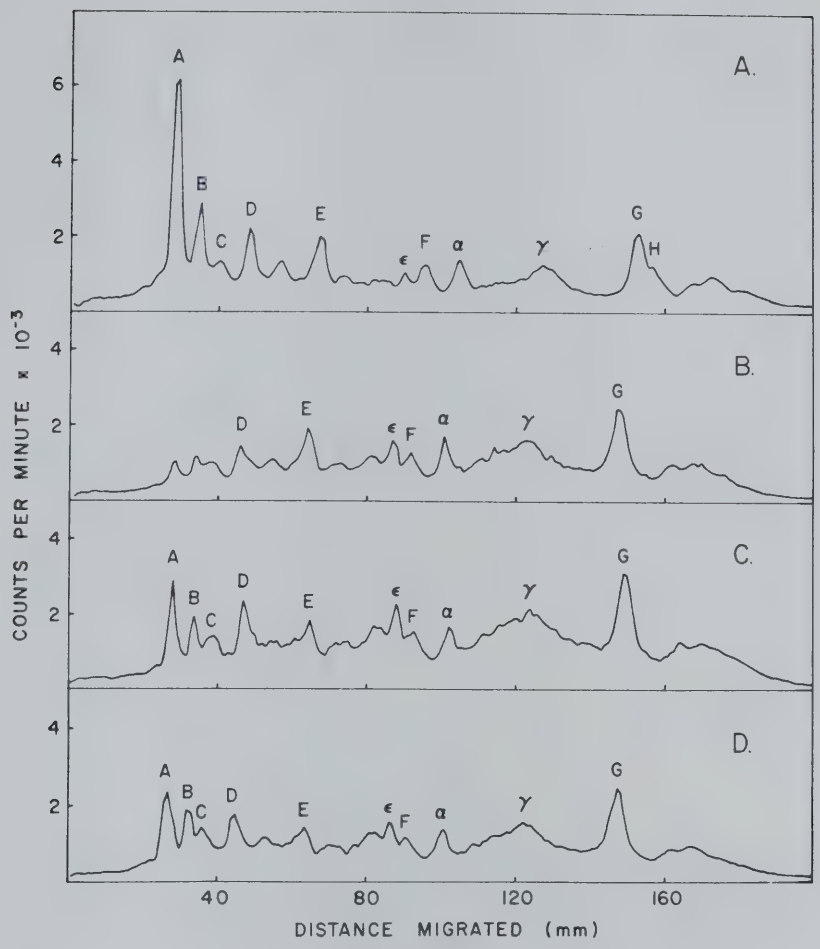


Figure 24. *In vitro* cleavage of ts 135 viral polypeptide precursors. A cytoplasmic extract was prepared after ts 135-infected cells, at 7.5 hr postinfection, were pulse-labeled for 15 min with [^{3}H] amino acids (50 μCi/ml). The extract was then divided into four aliquots and analyzed by SDS-7.5% polyacrylamide gel electrophoresis (A) before incubation, (B) after 2 hr incubation at 33°, (C) after 2 hr incubation at 39°, and (D) after 2 hr incubation at 39° in the presence of two volumes of an unlabeled extract prepared from wt virus-infected cells.

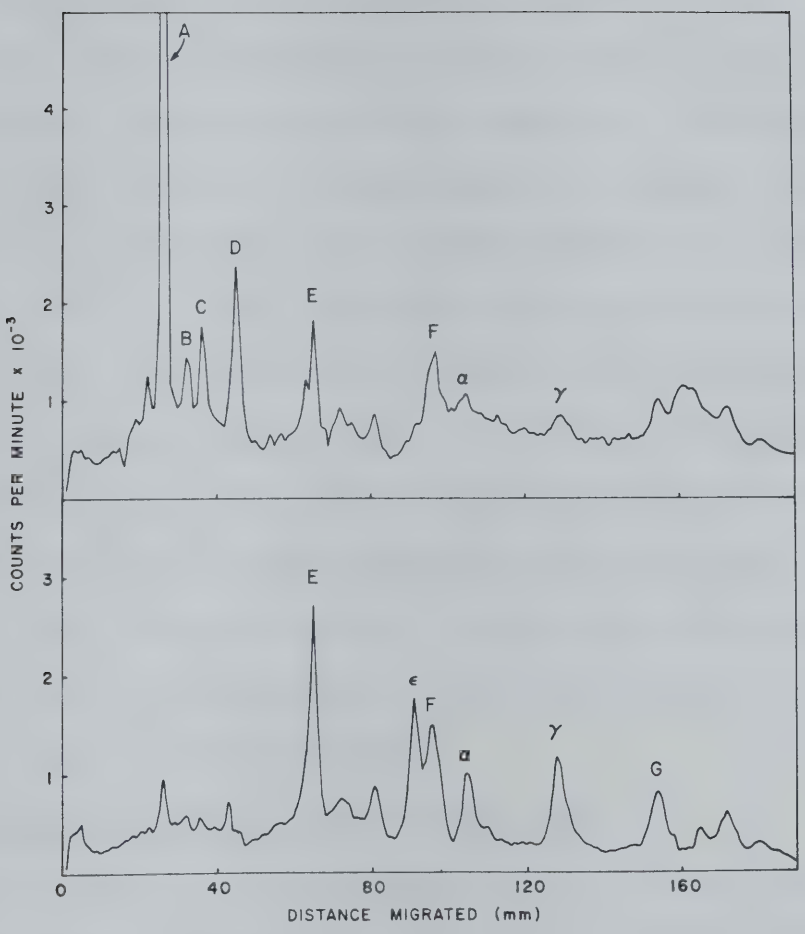


Figure 25. Electrophoresis in SDS-7.5% polyacrylamide gels of lysates of wt virus-infected cells, pulse-labeled for 15 min with [^{3}H]amino acids (50 μCi/ml) in the presence of cordycepin (200 μg/ml) at 4.75 hr postinfection at 37°. Upper panel: cells were lysed immediately after the labeling period. Lower panel: cells were lysed after a chase period of 90 min in the presence of cordycepin.

in cell monolayers incubated at 33° and 39°, and in cultures shifted from 33° to 39° at various times post-infection, were examined using the same protocal that was used for comparable studies with mutant ts 135. The results are shown in Figs. 26 and 27.

As was the case with mutant ts 135 (see Fig. 17) the growth curve obtained at 33° with mutant ts 520 is essentially identical to that obtained with wt virus at the same temperature, whereas little or no replication occurs at 39°. When ts 520-infected cultures are shifted from 33° to 39° at various times post-infection, virus replication is inhibited, but the shut-off is not as rapid as is the case with mutant ts 135. The results obtained from a study of viral RNA synthesis in ts 520-infected cells (Fig. 27) are essentially identical to those obtained from similar studies with mutant ts 135 (see Fig. 18). When infected cells are shifted from 33° to 39°, viral RNA synthesis is blocked rapidly, and apparently irreversible since it does not resume in cells that are returned to 33°.

Synthesis of Virus-Specific Polypeptides

An investigation of the synthesis and post-translational cleavage of virus-specific polypeptides in ts 520-infected cells showed that at 33°, the patterns of synthesis and cleavage do not differ from those observed in wt virus-infected cells (see Fig. 28 for illustrative electropherograms) and in ts 135-infected cells (see Fig. 22). However, when ts 520-infected cells were pulse-labeled at 39° and then incubated for an additional 90 min at 39° in the absence of labeled amino acids, the results obtained, and illustrated in Fig. 29, were found to differ significantly from those obtained with mutant ts 135. Whereas the cleavage of precursor proteins A and B is partially blocked at 39°

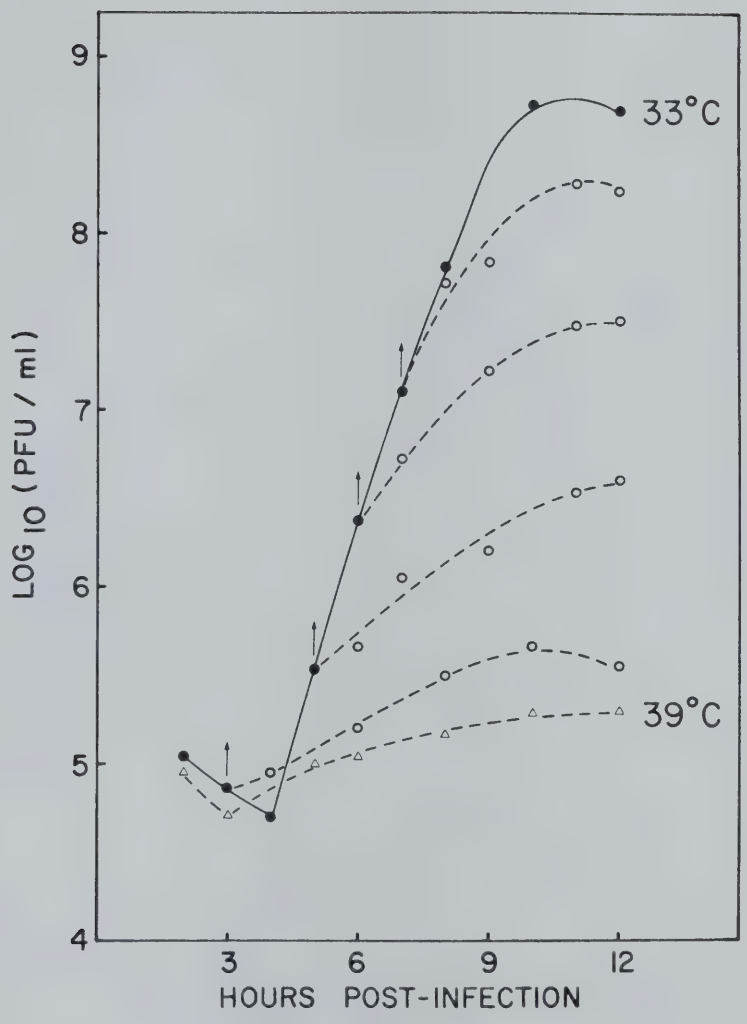


Figure 26. Growth behavior of mutant ts 520 at 33° and 39°. Virus production at 33° (●), at 39° (Δ), and after temperature shift-up from 33° to 39° (○) at times indicated by arrows.

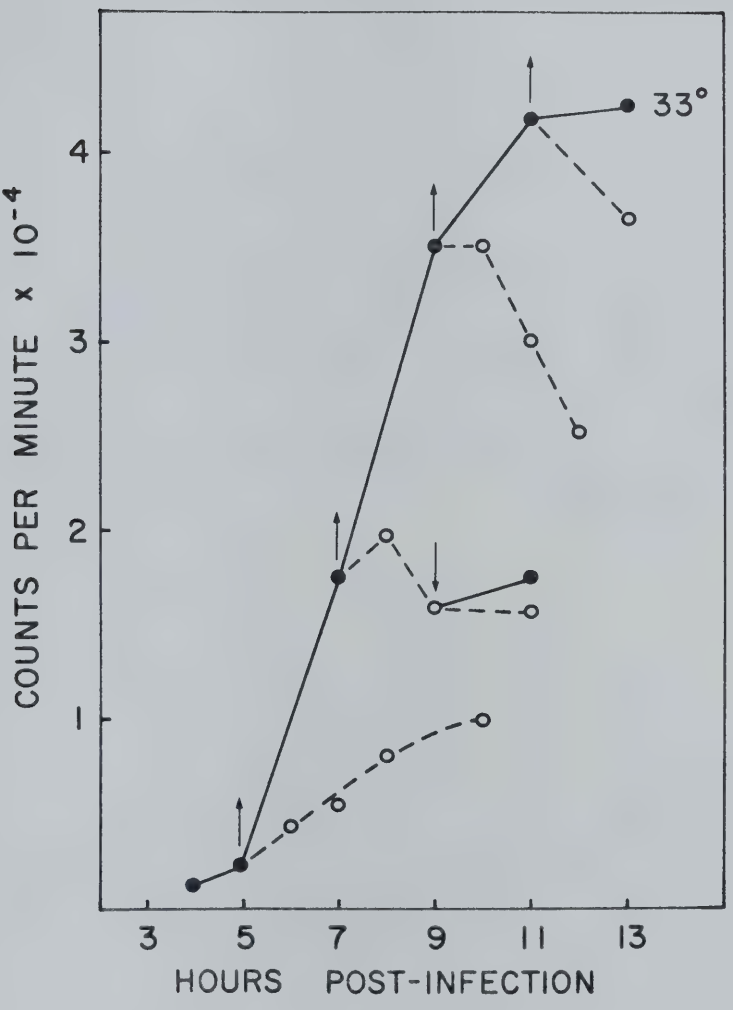


Figure 27. Effect of temperature on the synthesis of ts 520 viral RNA. ^{3}H-uridine (10 µCi/ml) was added to replicate cultures of infected cells at 2 hr postinfection. At various times thereafter, the cultures were harvested, the cells were lysed, and the lysates were analyzed for acid-insoluble radioactivity. (●) RNA synthesis at 33°, (○) RNA synthesis at 39°. The arrows indicate the times at which the cultures were shifted from 33° to 39° (↑) or from 39° to 33° (↓).

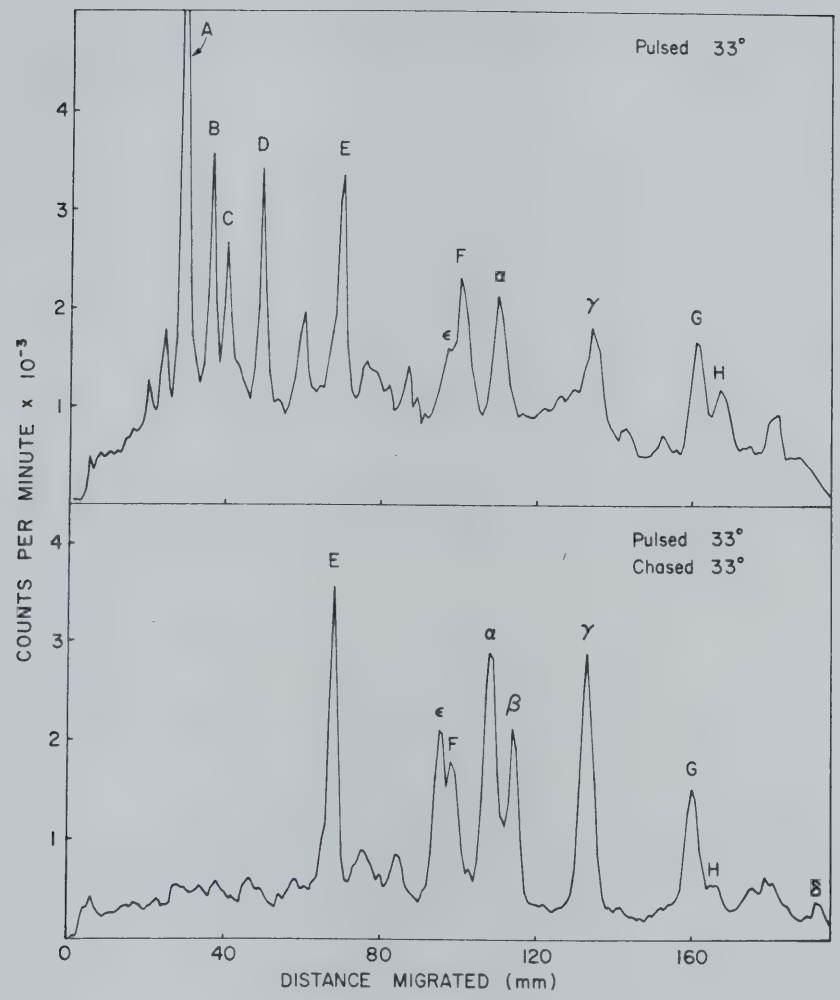


Figure 28. Electrophoresis in SDS-7.5% polyacrylamide gels of lysates of ts 520-infected cells, pulse-labeled for 15 min with ^{3}H-amino acids (50 μCi/ml) at 7.5 hr postinfection at 33°. Upper panel: cells were lysed immediately after the labeling period. Lower panel: cells were lysed after a chase period of 90 min.

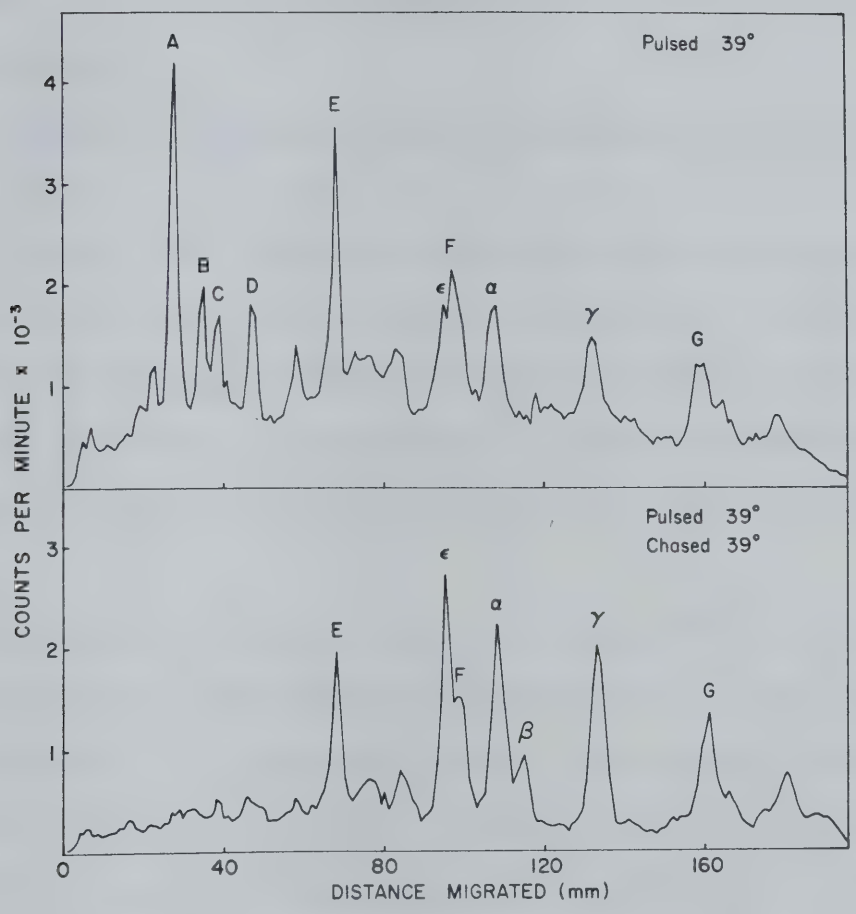


Figure 29. Electrophoresis in SDS-7.5% polyacrylamide gels of lysates of ts 520-infected cells, pulse-labeled at 39° for 15 min with [^{3}H] amino acids (50 µCi/ml) after the cells were shifted at 7 hr postinfection from 33° to 39° for 30 min. Upper panel: cells were lysed immediately after the labeling period. Lower panel: cells were lysed after a chase period of 90 min at 39°.

in ts 135-infected cells, the radiolabel that is incorporated into these proteins when ts 520-infected cells are pulse-labeled at 39° is chased completely into smaller viral polypeptides during a subsequent chase period at 39°. As would be expected for an RNA^- mutant, polypeptide ε was found to accumulate in ts 520-infected cells after a pulse-chase at 39°

C. Effect of Temperature on the Morphogenesis of *ts* 135 and *ts* 520

In Chapter III it was shown that 53S particles accumulate in wt virus-infected cells in the presence of cordycepin. Since RNA synthesis in both ts 135 and ts 520-infected cells is blocked at 39°, it was of interest to determine whether a similar accumulation of these subviral particles occurs when these cells are shifted from 33° to 39°.

Monolayer cultures of L cells, infected at 33° with the two mutants and with wt virus, were either 1) pulse-labeled for 15 min with [^{3}H] amino acids at 7.5 hr post-infection, and then incubated for an additional 90 min at 33° in medium free of labeled amino acids, or 2) shifted to 39° at 7 hr post-infection, pulse-labeled for 15 min at 7.5 hr post-infection, and chased for an additional 90 min at 39°. Cytoplasmic supernatants (S_{20} fractions) were then prepared from all cultures and analyzed by sucrose density gradient centrifugation. The results are presented in Fig. 30.

The sedimentation profiles obtained with S_{20} fractions prepared from ts 135-, ts 520- and wt virus-infected monolayers after a pulse and chase at 33° were found to be strikingly similar. All have a prominent virion peak (150S), and, as would be expected after a 90 min

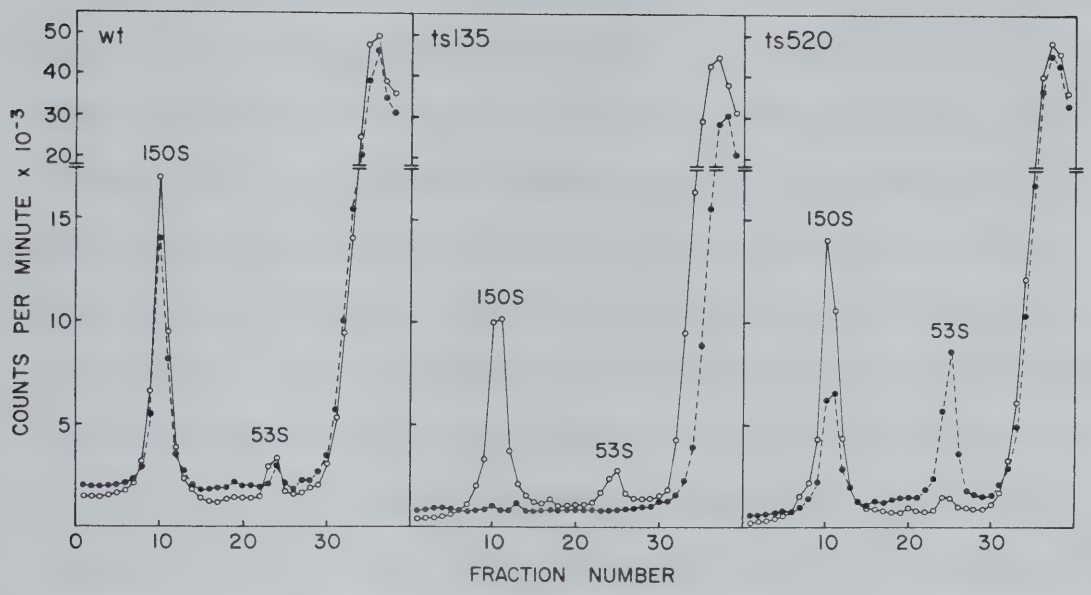


Figure 30. Sedimentation analysis of S_{20} fractions prepared from ts 135-, ts 520-, and wt virus-infected cells. Centrifugation through 15-45% sucrose gradients was for 13.5 hr at 20,000 rpm (Beckman SW 27.1 rotor). (○) S_{20} fractions were prepared after the cells were pulse-labeled for 15 min with [^{3}H] amino acids (50 μCi/ml) at 7.5 hr postinfection and then chased for an additional 90 min at 33°. (●) S_{20} fractions were prepared after the cells were shifted to 39° at 7 hr postinfection, pulse-labeled for 15 min at 7.5 hr postinfection, and chased for an additional 90 min at 39°.

chase, only a small peak of 53S particles. When the pulse and chase were carried out at 39°, the sedimentation profile obtained with the S_{20} fraction from wt virus-infected cells was essentially the same as that obtained when the manipulations were carried out at 33°, whereas in the case of ts 135-infected cells, no radiolabel was found in either the virion or 53S peak. With the S_{20} fraction from ts 520-infected cells, the amount of radioactivity in the virion peak was found to be sharply reduced relative to the amount present after a pulse-chase at 33°, and a significant accumulation of 53S particles was observed. It is interesting to note that these data confirm the conclusion drawn from earlier studies of the replication of mutants ts 135 and 520 (see Figs. 17 and 26), - namely that when infected cultures are shifted from 33° to 39°, the production of progeny virions is shut off more rapidly with mutant ts 135 than with mutant ts 520.

Discussion

Data obtained from the experiments described here suggest strongly that mutant ts 135 is a double mutant. When ts 135-infected cells are shifted from the permissive (33°) to the non-permissive (39°) temperature, the two temperature-sensitive defects are manifested by 1) a rapid and efficient shut-off of viral RNA synthesis, and 2) a partial block in the cleavage of structural polypeptide precursors A and B.

The RNA^- phenotype of mutant ts 135 could reflect either the inactivation of the virus-specific RNA polymerase at the non-permissive temperature, as is the case with some mutants of vesicular stomatitis virus (Szilágyi and Pringle, 1972), or a block in the synthesis of

the enzyme, as has been reported for a mutant of foot-and-mouth disease virus (Manor *et al.*, 1974). The results of the experiments in which the *in vivo* and *in vitro* stability of the ts 135-specific RNA polymerase were studied provide strong evidence that the enzyme is inactivated more rapidly at 39° than is the wt enzyme (see Figs. 20 and 21). However, the distinction between the two alternatives cited above may not be very clear in the case of picornaviruses. The weight of evidence suggests that, with these viruses, the virus-specific RNA polymerase is a complex of one virus-coded polypeptide and several, as yet unidentified, cellular polypeptides. If this is so, a temperature-sensitive defect in the virus-coded component (believed to be polypeptide E) could result not only in the inactivation at 39° of preformed enzyme, but in the inhibition of assembly of the enzyme complex at the non-permissive temperature.

Why viral RNA synthesis does not resume when cells infected with either of the RNA^- ts mutants are returned to 33° after being held at 39° for 2 hr is not clear from the data presented here. The failure to do so could reflect either the degradation of viral RNA during the 2 hr incubation at 39°, or the immobilization of viral RNA templates by binding to viral RNA polymerase molecules that have been irreversibly inactivated at the non-permissive temperature.

The second temperature sensitive defect exhibited by mutant ts 135, namely the partial block in the cleavage of structural polypeptide precursors A and B at 39°, could reflect either 1) a mutation in the putative virus-coded protease believed to be responsible for the post-translational processing of polypeptide A, or 2) a mutation in the precursor itself, which - presumably by causing a conformational

change in the precursor molecule - makes the precursor at least partially refractory to cleavage by the appropriate enzyme(s) at 39°.

Although the first of these possibilities cannot be ruled out entirely, the second is the far more likely in the light of the experimental evidence. First, the addition of a cytoplasmic extract prepared from wt virus-infected cells, which presumably contains a normal cleavage enzyme, does not enhance the *in vitro* cleavage of ts 135-specific polypeptides A and B (see Fig. 24). Second, the low thermal stability of mutant ts 135 is compatible with the proposition that it contains one or more altered structural polypeptides. The possibility that the cleavage defect exhibited by mutant ts 135 may be simply a secondary effect of the inhibition of RNA synthesis at the non-permissive temperature, seems to be ruled out by the observations that post-translational cleavages proceed normally in wt virus-infected cells in the presence of cordycepin, and in ts 520-infected cells at 39°. The observation that the cleavage of ε to β + δ is seen neither in cells infected with either of the two RNA^- ts mutants at 39°, nor in wt virus-infected cells in the presence of cordycepin, is consistent with the belief that this cleavage occurs only when the viral genome is packaged during the final stages of virion assembly.

The results of analyses of S_{20} fractions, prepared from mutant and wt virus infected cells after pulse-labeling and chasing at both the permissive and non-permissive temperatures are revealing. The observation that 53S particles accumulate in ts 520-infected cells when the latter are pulse-labeled and chased at 39°, is strikingly

similar to that made with wt virus-infected cells in which RNA synthesis is blocked by cordycepin (see Chapter III), and provides strong support for the hypothesis that the 53S particle is an intermediate in the assembly of Mengo virions. The observation that no radioactivity is found either in progeny virions or in 53S particles when ts 135-infected cells are pulse-labeled and chased at 39° is consistent with the evidence that this mutant contains defective structural polypeptides (and precursors thereof), and suggests that this lesion not only accounts for its low thermal stability, but prevents its normal assembly at the non-permissive temperature.

CHAPTER V

PROSPECTS FOR FUTURE RESEARCH

Morphogenesis of the Mengo Virion

The detection and characterization of a 53S particle in Mengo virus-infected cells, and the observation that this particle can be converted *in vitro* to one having a sedimentation coefficient of 75S, have provided the basis for a proposed scheme for the assembly of Mengo virions. The validity of the scheme, which differs significantly from those proposed earlier for poliovirus and for other members of the cardiovirus subgroup, depends in large measure on the accuracy of the estimated molecular weights of the 53S and 75S particles. These were calculated from sedimentation coefficients and from Stokes radii as determined by exclusion chromatography on Sepharose 4B. Although there seems to be no reason to doubt the validity of this method, it would be highly desirable to determine the molecular weights of both particles by a second, independent method, - the most obvious one being that of equilibrium sedimentation using the analytical ultracentrifuge.

In order to estimate molecular weights from analytical ultracentrifuge measurements, it would be necessary to obtain suspensions of highly purified particles containing of the order of 0.1 to 1 mg protein/ml. As isolated by sucrose density gradient centrifugation of either S_{20} or P_{45} fractions, the 53S particles are heavily contaminated with ribosomes and/or ribosomal subunits. They can be separated from these contaminants by isopycnic centrifugation in a CsCl density gradient, but are converted to 75S particles in the process. However,

it should be possible to purify the rather unstable 53S particles by either 1) treating the 53S fraction with ribonuclease(s) under controlled conditions to degrade ribosomal structures followed by a second sucrose density gradient centrifugation, or 2) affinity chromatography using antibodies against the capsid polypeptides of the Mengo virion as the ligand. Whichever approach turned out to be the most feasible, a serious logistical problem would remain in that a large number of cells (upwards of 10^{10}) would have to be processed to provide sufficient purified particles for analytical ultracentrifuge studies. Electron microscopic examination of purified particles could be revealing in that it would provide useful information regarding the size and shape of the particles.

If satisfactory methods for the purification of 53S and 75S particles could be developed, an intriguing project (albeit one with a low probability of success) would be to attempt to achieve the *in vitro* assembly of Mengo virions from viral RNA + either 53S or 75S particles + 14S particles. To give one's imagination free reign, one could conceive of such studies distinguishing between assembly schemes A and B (Fig. 16), if, for example, assembly were obtained with one or the other, but not both, of the 53S and 75S particles. Were *in vitro* assembly achieved, it would be possible to carry out the reaction with ^{3}H-labeled 53S and ^{14}C-labeled 14S particles, and, from analyses of the completed virions, to determine the molar ratio of the two particles in the virions and to ascertain whether the uncleaved ε chains are derived from one or the other, or both, of these subviral particles.

To return to more realistic projections, it would clearly be of

interest to extend the studies described here to other members of the cardiovirus subgroup of picornaviruses, as well as to members of other subgroups of this family. It would be of particular interest to determine precisely the molecular weight of the 73S particle found in poliovirus-infected cells, to see if it does in fact correspond to a complete empty capsid [$(VP0,1,3)_{60}$] as has been assumed, or whether it is equivalent to the 75S particle [$(\varepsilon\alpha\gamma)_{50}$] described herein.

It would also be of interest to examine the early stages of the morphogenesis of Mengo virus with the objective of identifying the immediate precursor of the 14S particle, - the one common denominator in the assembly pathway of all picornaviruses. Is that precursor A_5, as has been proposed for EMC and rhino viruses, or is it a 5S particle, ($\varepsilon\alpha\gamma$), which would correspond to the 5S, (VP0,1,3), particle believed to be the precursor of the 14S particle in the poliovirus system?

Studies of Temperature-Sensitive Mutants of Mengo Virus

The ts mutants of Mengo virus isolated in this laboratory were isolated in the first instance in the hope that by defining the precise biochemical lesion associated with each, some insight might be gained regarding the biological functions of the non-structural viral polypeptides. Viewed with this objective in mind, the studies of the two RNA^- ts mutants described in this thesis have not been fruitful. However, they do help to indicate the direction of future studies.

It would be a logical point of departure to carry out a careful study of the synthesis of viral-specific polypeptides in cells infected with a large number of the available ts mutants, using the technique of SDS-PAGE. Of particular interest would be an examination of the fate of proteins synthesized during a brief labeling period (at either

33° or 39°) during a subsequent chase at 39°. Knowing the number and molecular weights of all virus-specific polypeptides synthesized in Mengo-infected cells, as well as the pattern of post-translational cleavages by which the stable, viral gene products are formed, it would be relatively easy to detect any deviations from the normal picture. Since the technique of SDS-PAGE resolves proteins solely on the basis of size, it is likely that with many mutants, no significant change in the normal pattern would be seen. However, any interference (at 39°) with the normal pattern of cleavage - which could result from either a ts mutation in the hypothetical viral-specified protease, or from an amino acid replacement at a critical position in a precursor polypeptide - should be readily detectable.

These studies would not, of course, be particularly helpful in identifying the viral polypeptide that carried the ts mutation, - especially in those cases where the cleavage pattern was normal. However, it could be profitable to couple these studies with studies of the virus-specific polypeptides in ts mutant-infected cells using the two dimensional electrophoresis system developed by O'Farrell (1975). This system separates proteins by isoelectric focusing in one dimension, and according to molecular weight by SDS-PAGE in the second dimension. Since the system can resolve proteins differing by a single charge, it is potentially capable of detecting a mutant polypeptide, if the mutation is the result of the replacement of a charged by an uncharged amino acid (or vice versa). If, in cells infected with an RNA^- mutant, the only viral polypeptide exhibiting an abnormal behavior was E, it would provide strong presumptive evidence that E is the viral-coded component of the viral RNA replicase.

If polypeptide F was the only altered viral polypeptide in cells infected with a cleavage mutant, it would suggest that F is the viral-coded protease. It is probably not over-optimistic to expect that by screening a number of ts mutants in this way, it would be possible - in at least some cases - to identify the viral polypeptide that carries the ts mutation.

CHAPTER VI

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